ABSTRACT

Title of Document: **FOOD SAFETY IMPLICATIONS OF BIOFILMS FORMED BY RESIDENT BACTERIA IN FRESH-CUT PROCESSING ENVIRONMENTS**

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Biofilms on equipment surfaces can be vectors for cross-contaminations in food processing facilities. A particular problem is that biofilms can protect pathogenic bacteria from daily cleaning and disinfection operations. In the present study, microflora were recovered from fresh-cut processing facilities, identified, and tested for biofilm forming potential. Subsequently, dual-species biofilms of selected isolates and *Escherichia coli* O157:H7 were investigated. Approximately 30% of the isolates were potent biofilm formers, producing large amounts of biomass. A hundred and seventeen tested isolates were identified into 23 genera, including plant related bacteria and coliforms with some opportunistic pathogens. Dual-species biofilms formed by *Burkholderia caryophylli* or

Ralstonia insidiosa and *E. coli* O157:H7 manifested increased biomass in comparison to their monocultures. Additionally, about a one-log unit increase of *E. coli* O157:H7 cell counts were observed for both dual-species biofilms. To test the effects of environmental factors on growth of *R. insidiosa* and *E. coli* O157:H7 in dual-species biofilms, factors tested included low temperature (10 ^{o}C), media with different composition of nutrient sources (10% TSB, M9, 1.25% Cantaloupe Juice) and a continuous culture system with limited nutrients. *E. coli* O157:H7 cell counts increased for all tested conditions. To examine bacterial localization within biofilms, confocal laser scanning microscopy (CLSM) and transmission electron microscopy (TEM) were used. Images showed distinct spatial distributions with *E. coli* O157:H7 commonly located at the bottom and also interspersed among *R. insidiosa* cells. To test the interactions with other pathogens, *R. insidiosa* was co-cultured with *Listeria monocytogenes*, *Salmonella* spp., and Shiga toxin-producing *E. coli*. Cells counts for 7 out of 9 tested pathogenic bacteria strains were increased (0.36-1.60 CFU log units). It is notable that the biomass formed by *R. insidiosa* and *L. monocytogenes* was much greater than those produced by other tested combinations. These results indicate that *R. insidiosa* could be a food safety risk in fresh-cut processing environments by providing protective habitats for pathogenic bacteria.

FOOD SAFETY IMPLICATIONS OF BIOFILMS FORMED BY RESIDENT BACTERIA IN FRESH-CUT PROCESSING ENVIRONMENTS

By

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biofilm; ■ *R. insidiosa* in dual-species biofilms. Two-way ANOVA was performed to elucidate the effects of culture method (mono- or dual-species biofilms) and incubation time on the numbers of *E. coli* O157:H7 or *R. insidiosa*, respectively. Asterisk represented significant difference between the cells counts of *E. coli* O157:H7 in monoculture and dual-species biofilms ($p<0.05$).

Figure 6.2 Cell counts of *E. coli* O157:H7 and *R. insidiosa* in biofilms developed using drip flow biofilm reactor following 3 days incubation at room temperature. Initial inoculation in TSB was 10^7CFU/ml for *E. coli* O157:H7 strain FS4052 and *R. insidiosa* strain FC1138 followed by continuous culture with 1% fresh TSB. □ Monoculture biofilm; ▭ dual-species biofilms. Asterisk represented significant difference between the cells counts of *E. coli* O157:H7 in monoculture and dual-species biofilms ($p<0.05$).

Figure 6.3 CLSM images showed the development of dual-species biofilms formed by *E. coli* O157:H7 strain FS4052 and *R. insidiosa* after co-inoculation for 4h, 40h and 72 h. Cells were stained with red fluorescent dye SYTO 61. *E. coli* O157:H7 cells remained green due to strong expression of GFP. For each image, main panel showed the top cut-view of the biofilms and the side panels showed cut-views of a cross section of the biofilms.

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Figure 7.1 Total bacteria counts of tested foodborne human pathogen in monoculture and dual-species biofilms produced with *R. insidiosa*. □ Monoculture biofilm grown for 1 day; ■ Dual-species biofilms grown for 1 day. Student's T-test was used to elucidate the statistical relationship between the two columns (* $p<0.05$).

Figure 7.2 Total cell counts of *E. coli* O157:H7 strain FS4052 in monoculture and dual-species biofilms formed with/without the presence of *R. insidiosa*. ○ Monoculture of *E. coli* O157:H7 biofilm; ● *E. coli* O157:H7 in dual-species biofilms followed by co-inoculation; ▲ dual-species biofilm formed by inoculating *E. coli* O157:H7 in 1 day pre-formed *R. insidiosa* biofilm. One-way ANOVA was used to test the effect of different culture on the presence of *E. coli* O157:H7 in biofilms. Tukey's multiple comparison test was used to compare the *E. coli* O157:H7 counts in different biofilms sampled at the same time point ($p<0.05$).

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Figure 7.4 Total aerobic counts of *E. coli* O157:H7 strain FS4052 in monoculture biofilm formed in cell free pre-conditioned TSB after 24 h incubation. (A) in TSB; (B) in

R. insidiosa supernatant; (C) in *E. coli* O157:H7 supernatant; (D) in mixed culture of *R. insidiosa* and *E. coli* O157:H7. One-way ANOVA was used to test the effect of different pre-conditioned media on *E. coli* O157:H7 biofilm formation. Tukey's multiple comparison test was used to compare the *E. coli* O157:H7 counts in biofilms grown in different culture media ($p<0.05$).

Figure 7.5 Total cell counts of *E. coli* O157:H7 strain FS4052 in monoculture biofilm (A) and biofilm formed on heat-inactivated *R. insidiosa* biofilm (B). Student's T-test was used to compare the *E. coli* O157:H7 counts in different biofilm samples ($p<0.05$).

Figure B. SEM images of mono and mixed-culture biofilm formed by *R. insidiosa* and dual-species biofilms with *E. coli* O157:H7 (FS4052). (A) Monoculture 3 d *R. insidiosa* biofilm; (B) 3 d dual-species biofilms.

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Figure E. Total biomass production of 1-day *R. insidios*a biofilm formed on glass slide in drip flow biofilm reactor before heat treatment (A) and after heat treatment (B). Serial dilution was made to keep the optical density reading between 0.2-0.8.

Chapter 1: Introduction

The Center of Disease Control and Prevention (CDC) reported that each year one in six Americans (approximately 48 million) are affected by foodborne illnesses and there are 3000 deaths due to the consumption of tainted food products (CDC, 2011a). Produce is recognized as a major vector of foodborne outbreaks and is the source of the second largest number of illnesses among all food commodities (CDC, 2011b). The overall cost of illnesses in the United States related to produce is approximately 1.4 billion dollars per year (Morris Jr et al., 2011). Furthermore, the consumption of fresh produce has been increasing in recent years (ERS, 2012; FDA, 2001; Kaufman et al., 2000). To meet this demand, industry uses large centralized production facilities. One of the major concerns is that centralized production can increase the risk of cross-contamination due to enlarged processing volume. Additionally, the risks associated with contaminated products may be amplified because of the extended distribution areas that result from using centralized processing facilities (FDA, 2001; Roever, 1999). It has been hypothesized that biofilms can be vectors for cross-contamination during processing by providing protective environments for pathogenic bacteria. In order to elucidate the role of biofilms in fresh-cut processing facilities, it would be valuable to study the biofilm forming potentials of native microflora and their interactions with foodborne pathogenic bacteria.

Biofilms formed in food processing plants can create habitats for pathogenic bacteria and be a source of cross-contamination (Midelet and Carpentier, 2004; Silagyi *et al.*, 2009). Bacterial biofilms are generally aggregated complexes that can include multiple microorganisms as well as dirt and debris. The complexes generally form on solid substrata and include an external barrier of extracellular polymeric substances that

protects the complexes from adverse environmental impacts. In particular, pathogenic organisms embedded in biofilms are protected from cleaning and sanitation efforts (Burmolle et al., 2006; Jefferson, 2004). Experimentally, it has been demonstrated that the growth of pathogenic bacteria can be enhanced in dual-species biofilms containing resident bacteria (Carpentier and Chassaing, 2004; Christensen et al., 2002; Hansen et al., 2007; Simoes et al., 2007). For example, when co-cultured with *Acinetobacter calcoaceticus* (Klayman *et al.*, 2009) or *Pseudomonas aeruginosa* (Habimana, Heir, et al., 2010; Klayman et al., 2009), *Escherichia coli* O157:H7 exhibited an increased biovolume in the resulting biofilms. Pathogenic bacteria in biofilms can be transferred to food commodities. For instance, more than 10^3 CFU/cm^2 *E. coli* O157:H7 were transferred from a monoculture biofilm to fresh produce products when the produce was allowed to contact the biofilm for 30 min (Silagyi *et al.*, 2009). Alternatively, wash water can transfer pathogenic bacteria released from biofilms to other equipment surfaces or fresh produce resulting in cross-contaminations (Gil *et al.*, 2009).

Studies demonstrated that native microflora recovered from food processing facilities, such as meat and dairy plants, are capable of forming biofilms (Chmielewski and Frank, 2003); however, only a few studies focused on fresh-cut processing facilities. Kaneko and colleagues (1999) reported that heavy contamination (>10^5 CFU/log cm^2) was found on various processing equipment surfaces after cleaning operations in ready-to-eat fresh vegetable processing facilities. Van Houdt and colleagues (2004) reported that most of the resident bacteria recovered from raw vegetable processing facilities were able to form biofilms, and three species were identified, including *Vibro diazotrophicus*, *Serattia plymuthica* and *Pantoea agglomerans*. In summary, there is a lack of knowledge

of the major bacterial species that are commonly resident in fresh-cut processing facilities and the role of these bacteria in forming biofilms that might provide a protective environment to pathogenic bacteria.

Chapter 2: Research Objectives

The goal of this research is to recover resident bacteria from fresh-cut processing facilities and evaluate the biofilm forming capacities of the isolates. Dual-species biofilms formed by resident bacteria strains and *E. coli* O157:H7 are characterized by biomass production and cell counts. A selected isolate (*Ralstonia insidiosa*) with potent biofilm forming capacity is used to study the impact of environmental conditions on dual-species biofilm formation, as well as its ability to form dual-species biofilms with *Listeria monocytogenes*, *Salmonella* spp., and Shiga toxin-producing *E. coli*. Initial studies are conducted to elucidate factors that might be expected to influence the growth of *E. coli* O157:H7 in dual-species biofilms formed with the selected resident bacteria, including a pre-formed biofilm of resident bacterium, co-aggregates, extracellular compounds, and a biomatix without vital resident bacteria.

Objective #1: Capture, recover, isolate, and identify resident bacteria in fresh-cut produce processing facilities and test the biofilm-forming capacity of each isolate.

Objective #2: Quantify the total biomass and enumerate the total aerobic counts of the resident bacteria and *E. coli* O157:H7 in monoculture and dual-species biofilms. Select one resident bacterium with potent biofilm production for further study (Objective #3 and #4).

Objective #3: Grow dual-species biofilms with resident bacterium and *E. coli* O157:H7 under selected environmental conditions, including low temperature, different types of limited nutrient source, and continuous limited nutrient flow.

Objective #4: Characterize dual-species biofilms formed by selected resident bacterium and *Listeria monocytogenes*, *Salmonella* spp., and Shiga toxin-producing *E. coli* by using methods described in Objective #2.

Objective #5: Enumerate *E. coli* O157:H7 counts in biofilms formed with a pre-formed resident bacterium biofilm, in the supernatant after 24 hrs incubation of resident bacterium, and on a heat inactivated resident bacterium biofilm. Examine intergeneric bacteria adherence between selected bacterium and *E. coli* O157:H7.

Chapter 3: Literature Review

3.1 Food safety related to fresh-cut produce

3.1.1 Fresh-cut produce related outbreaks

On January 2011, Food Safety Modernization Act (FSMA) was signed into law. Its purpose is to ensure food safety based on a preventative and risk-based system. A special emphasis has been given to fresh produce products due to their increasing number of foodborne outbreaks in recent years (ERS, 2012). An analysis conducted using data collected over the past 11 years suggested that produce resulted in approximately $ 1.4 billion annual cost of illness, which is ranked number 4 among all food commodities (Morris Jr *et al.*, 2011). Moreover, produce associated outbreaks hold the highest average number of illness (48 cases/outbreak) than other food commodities, such as poultry (30 cases/outbreak), beef (27 cases/outbreak), or seafood (10 cases/outbreak) (Scharff, 2010). Within all the outbreaks, several pathogen-food combinations are commonly associated with outbreaks (Morris Jr *et al.*, 2011). *Escherichia coli* O157:H7-fresh cut leafy green is one of the well recognized combinations (HHS, 2005). By analyzing the data published by CDC from 2003-2007, a study showed that 39% of foodborne outbreaks and 54% foodborne illnesses associated with *E. coli* O157 were attributable to produce products (Scharff, 2010). Therefore, elucidating the potential contamination pathways of *E. coli* O157:H7 on fresh-cut products in order to mitigate the risks are key steps in providing trustworthy and safe products for public consumption.

3.1.2 Bacteria on fresh produce

3.1.2.1 Native microflora on fresh produce

Fresh produce is a common host of numerous microorganisms, including beneficial and occasionally pathogenic strains. The bacterial population on different types of fresh produce is highly variable (Roever, 1999). Romaine lettuce had highest native bacteria population of 7.0 log CFU/g, followed by baby carrot, green bell pepper, and alfalfa seeds with 4.0-5.0 log CFU/g (Liao and Fett, 2001). The packaged red leaf "Lollo Rosso" lettuce and Romaine lettuce had a bacteria population at 6.0 log CFU/g after processing followed industrial practice (Allende *et al.*, 2004; Luo, 2007).

Many phytobacteria have been isolated from fresh produce related products, including *Bacillus* spp., *Enterobacter asburiae, Klebsiella* spp., *Pseudomonas* spp., *Erwinia* spp., *Rhanella* spp., *Xanthomonas* spp., *Flavobacterium* spp., and lactic acid bacteria (Cruz et al., 2008; Liao and Fett, 2001; Morris and Monier, 2003; Rudrappa et al., 2008; Schuenzel and Harrison, 2002). Among all of the plant associated bacteria, *Pseudomonas* spp. is the most common genus isolated from vegetables and dominates the bacterial population by up to 50-90% (Zagory, 1999). Although most of the listed microorganisms are harmless to human being, it is reasonable to postulate that they can become part of the resident microflora in fresh-cut processing facilities.

3.1.2.2 Foodborne human pathogens on fresh produce

It has been shown that bacterial pathogens such as *E. coli* O157:H7 and *Salmonella* can contaminate fresh produce such as leafy greens, alfalfa sprouts, and cantaloupes via various routes (Delaquis et al., 2007; Doering et al., 2009; Fan et al., 2009; Kroupitski, Pinto, et al., 2009). Warner and colleagues observed the slimy 3 dimensional aggregations of *Salmonella* Thompson on spinach leaf surface around open stomata (Warner *et al.*, 2008). Similar results were reported with *E. coli* O157:H7

attachment on iceberg lettuce leaves, where cut edges were more preferable for the attachment than the intact leaf surfaces (Seo and Frank, 1999).

After attachment, bacterial internalization for leafy greens can be triggered by light and chemotaxis (Kroupitski, Golberg, et al., 2009). *E. coli* O157:H7 was found to have penetrate 20-100 mm below the surface in stomata and cut edges of lettuce leaves. The internalized *E. coli* O157:H7 showed enhanced resistance against disinfection treatments (Seo and Frank, 1999; Takeuchi and Frank, 2000). Additionally, the *E. coli* O157:H7 survived for up to 10 days when stored at refrigerator temperature (4 ^{o}C) (Lopez-Velasco *et al.*, 2010). During this storage period, *E. coli* O157:H7 could increase the expression of virulent genes, such as *stx2A* and *fliC* (Carey *et al.*, 2009), indicating a higher risk of occurrence of foodborne outbreaks (Harris *et al.*, 2003).

3.1.3 Resident bacteria in fresh-cut processing facilities

To minimize the microflora population on fresh-cut products and ensure product safety, cleaning and sanitation operations and Cleaning In Place (CIP) practices are widely used for both food contact and non-food contact surfaces in processing facilities (Gil *et al.*, 2009). For surface cleaning purposes, commercial disinfectants are designed to remove different types of food residuals, such as carbohydrates, fats, proteins and minerals, etc., on various types of surface materials. Generally, cleaning practices have 5 steps, including food debris removal, rinsing, application of detergent, post-rinsing, and application of sanitizer. Detergents widely used on equipment surfaces in food processing facilities mainly include chlorine-, quaternary ammonium-, acid- and alkaline-based compounds (Simoes *et al.*, 2010b).

Of note, the primary use of disinfection is to prevent microbial transfer among produces through out washing steps due to the accumulated microbial populations in reused wash water (Gil *et al*., 2009). For fresh-cut produce, an ideal sanitizer should be efficient at killing microorganisms, while maintaining the qualities and sensory values of the final products (Simoes *et al.*, 2010b). A large number of physical, chemical or combined methods have been tested for the efficacy on inactivation of pathogens on fresh produce in laboratory (Artés *et al.*, 2009; Gil *et al.*, 2009). Although many of these showed promise, chlorine-based water is still the method most commonly used to reduce microorganisms on fresh produce. There are limitations to using chlorinated water on removing microbial contaminations. Active hypochlorite, the functional molecule that kills bacteria, can easily lose its effectiveness in the presence of nitrogen containing compounds, including organic materials from produce exudates (Inatsu *et al.*, 2007). In general, the chloride-based treatment could only achieve 2-log reductions of natural microflora attached to produce products (Allende *et al.*, 2007; Inatsu *et al.*, 2007).

Different from produce, equipment surfaces in processing facilities can be treated by various disinfectants at high dose in order to effectively mitigate microbial growth. However, there are places that are hard to clean, which might result in microbial accumulation and attachment at those local areas. On average, current cleaning and sanitation practices only achieve 90% reduction in microorganisms in multiple food processing industries (Chmielewski and Frank, 2003; Gibson et al., 1999b; Norwood and Gilmour, 2000; Wirtanen et al., 1996). Therefore, microorganisms that remain in processing facilities after cleaning and sanitation practices are recognized as resident bacteria (Marouani-Gadri *et al.*, 2009).

3.1.4 Potential biofilm formation on abiotic surfaces

In fresh-cut processing facilities, resident bacteria can attach to equipment surfaces, switch to sessile living status, and eventually form biofilms. Studies suggested that most bacteria naturally grow with other microbial partners in forming biofilms (Feazel et al., 2009; Welch et al., 2005). Pathogenic bacteria could potentially co-exist with various environmental bacteria and be sheltered in multispecies biofilms against environmental stresses (Castonguay *et al.*, 2006). Integrated pathogens exhibit higher resistance to antimicrobials and tolerance to poor nutritional environments compared to their planktonic counterparts (Van der Veen and Abee, 2011). This possibility may lead to cross-contamination of fresh produce via protecting pathogenic pathogens from the cleaning and disinfection efforts and transferring the pathogenic pathogens to clean products.

3.2 Biofilm formation in fresh-cut processing facilities

3.2.1 Biofilm life cycle and major properties

Biofilm is generally an aggregated complex community of microbial species that typically adhere to solid substrata and exists in diverse environments. Biofilm formation is a bacterial living strategy in response to environmental stresses (Jefferson, 2004). In most cases, bacterial biofilm formation seems to be induced by cellular stresses or by suboptimal environmental conditions. The potential environmental stresses include limited nutrient availability, low temperature, changes in osmosis and pH, oxygen radicals and high metal concentrations (Jefferson, 2004; Landini, 2009).

In stressful environments, when bacteria get close to solid substrates, they tend to adhere on surfaces to form biofilms (O'Toole *et al.*, 2000). The initial step of biofilm

formation is cell adherence, which is assisted by different classes of adhesins that appear on cell surfaces (Van Houdt and Michiels, 2005). Bacteria that primarily attach to surfaces form a monolayer biofilm, which create microniches for other planktonic bacteria to anchor and thus form multispecies biofilms. As biofilms mature, accumulated metabolites can alter microenvironments. The sessile cells show less aerobic respiration and more exopolysaccharide production (Karatan and Watnick, 2009; Stewart and Franklin, 2008). Meanwhile, intergeneric communication takes place with transcription of quorum sensing molecules and other molecules evolved in metabolic pathways. In nature, these changes produce multispecies biofilms with complex matrix structures (O'Toole *et al.*, 2000). At the late stage of biofilms maturation, a small portion of bacteria can switch their living status from sessile to swimming in the biofilm structure (Verstraeten *et al.*, 2008). With EPS degradation, these active bacteria can detach from the biofilm and return to planktonic growth (Ma *et al.*, 2009).

Typically, mature biofilms are highly structured and organized communities. The heterogeneity of biofilm structure depends on the bacteria characteristics and resulting changes in microenvironments within the biofilms. Some bacteria produce biofilms with mushroom-like structures with valleys in between, whereas the others form compact biofilms with smooth surfaces (Bridier, Tischenko, et al., 2011). Current thought is that the mushroom-like structures are related to the production of extracellular polymeric substances (EPS), which adds complexity to the biofilm structure (Stoodley *et al.*, 2002). In many cases, cell-free areas, named “hollow voids”, have been observed in the mushroom-like structures (Bridier *et al.*, 2010). These highly structured hollow voids maintain the motile bacteria that switch inversely from sessile living status (Karatan and

Watnick, 2009).These structural characteristics have been widely found in both Gram-positive and Gram-negative bacterial biofilms, indicating that it may be a prevailing process in the life cycle of biofilms.

There are two major properties often expressed during biofilm maturation, extracellular polymeric substrates (EPS) production and enhanced antibiotic resistance (O'Toole *et al.*, 2000). The EPS is mainly composed of exopolysaccharide, extracellular DNA, and proteins that have the potential to alter properties of the biofilm matrix (Flemming and Wingender, 2010). In contrast to planktonic bacterial counterparts, sessile cells in biofilms are well protected by EPS from environmental stresses. Bacterial antibiotic resistance can be up to 1000-fold higher than for planktonic bacteria (Jefferson, 2004). The enhanced resistance is not only due to the inability of antibiotics and sanitizers to penetrate EPS, but also by the ability of extracellular proteins to attenuate the effectiveness of active components of sanitizers (Flemming and Wingender, 2010). The slow growth rates in biofilms also contribute to lowering the sensitivity of bacterial cells to external stresses such as sanitizers and antibiotics (Karatan and Watnick, 2009). These factors also contributed to higher resistance to antimicrobial materials and increased tolerance to poor nutritional environments (Ihssen and Egli, 2004).

3.2.2 Environmental factors affecting biofilm formation

Daily cleaning and sanitation, and rigorous temperature control in fresh-cut processing facilities create stressful environments for resident bacteria (Capozzi *et al.*, 2009). The highly humid environments in processing facilities provide water needed for metabolism. After washing, cleaned fresh produce could carry a small amount of water on processing equipments thus create wet food-contact surfaces (Chmielewski and Frank,

2003). Leakage from chopped produce provides water-soluble nutrients for microorganisms. Moreover, localized temperature irregularities due to heat generated from equipments and ununiformed air-flow can favor of the growth of microorganism (Zagory, 1999). After sanitation, the disinfectant residual is often diluted by wash water, thus, creates suboptimal sanitizing conditions that can trigger biofilm formation (White-Ziegler *et al.*, 2008). All of these stresses have the potential of inducing the production of biofilms on abiotic surfaces, like utensils, processing equipments, *etc*.

The surface properties of the materials used in food processing facilities can be a critical factor for biofilm formation. Materials such as glass, rubber, stainless steel and various plastics that are widely used in food processing facilities can be the substrata for biofilm formation. Surface characteristics that influence the likelihood of biofilm formation included surface roughness, cleanability, disinfectability, wettability (determined by hydrophobicity) and vulnerability to wear (Bridier, Briandet, et al., 2011). The capacities to support biofilm formation of multiple materials used in food processing facilities have been studied (Meyer, 2003; Rogers et al., 1994). Van Houdt summarized that the ranking from high to low is high-density polypropylene, stainless steel, and glass (Van Houdt and Michiels, 2010).

3.2.3 Biofilm forming bacteria recovered from food processing environments

Biofilm forming bacteria have been recovered from various surfaces in meat and dairy processing facilities (Chmielewski and Frank, 2003; Kumar and Anand, 1998) and municipal water distribution networks (Simoes *et al.*, 2010a). For example, environmental bacteria *Bacillus* spp. and *Pseudomonas* spp., recovered from pipes in meat or dairy processing facility, could produce biofilm on abiotic surfaces, including

stainless steel and Teflon (Jeong and Frank, 1994; Wirtanen *et al.*, 1996). Moreover, the multispecies biofilms formed by resident bacteria recovered from dairy and meat processing facilities supported the growth of *L. monocytogenes* at 10 ^{o}C (Jeong and Frank, 1994). These findings suggest that resident bacteria in meat and dairy facilities are able to form both monoculture and multispecies biofilms.

Potential biofilm formation on abiotic surfaces has also been reported for produce facilities. Results acquired from a study conducted after a salmonellosis outbreak in the citrus-processing facility showed high microbial populations (5.0 log CFU/unit) and fecal coliforms on conveyor belts and surge belts after daily cleaning and disinfection operation (Parish, 1998). Similar heavy contamination was found in two ready-to-eat fresh vegetable processing facilities in Japan (Kaneko *et al.*, 1999). Additionally, it has been reported that most of the isolates from a raw vegetable processing line had a higher biofilm forming capacity than a laboratory *E. coli* strain that was used as a reference (Van Houdt *et al.*, 2004).

3.3 Physiological factors mediating biofilm formation in vitro

In response to environmental stresses, bacteria can undergo a series of physiological changes to adapt and form biofilms. In this section, *E. coli* biofilm lifecycle will be discussed as a model of bacterial biofilm formation due to the extensive studies that have been done with this bacterial species. Several key factors that mediate *E. coli* biofilm formation will be included (general stress response sigma factor, cell surface adhesins, EPS production, and quorum sensing).

3.3.1 General stress response sigma factor

The general stress response sigma factor RpoS, also known as alternative σ^S subunit of RNA polymerase, is a key component in a cell response to harsh environments (Hengge, 2008). In liquid culture, the expression of RpoS is activated after bacterial growth enters stationary phase (Hengge, 1999). As *E. coli* biofilm formed, RpoS is upregulated in *E. coli* O157:H7 cells (Lee *et al.*, 2011). With the accumulation of RpoS, a large number of RpoS dependent genes are upregulated. The expression of these genes leads to cell behaviors that can result in biofilm formation, including enhancement of cellular resistance to stresses, DNA protection, and production of extracellular structures (Lacour and Landini, 2004; Patten *et al.*, 2004; Weber *et al.*, 2005).

3.3.2 Cell surface adhesins

In the human gastrointestinal (GI) tract, *E. coli* O157:H7 first attaches to epithelial cells via a number of adhesins to start an infection. Similarly, cell adhesins also play an important role in the attachment of *E. coli* O157:H7 to abiotic surfaces to initiate biofilm formation. The adhesins are composed of extracellular organelle and cell membrane adhesins, both of which contribute to the initial attachment phase of biofilm formation (Van Houdt and Michiels, 2005). The extracellular organelle gives bacterial cells motility to overcome effects of repulsive forces between bacterial cells and abiotic surfaces. When the bacteria are close to the surface, cell membrane adhesins bind to the surface to further enhance attachment (Karatan and Watnick, 2009).

Among all cell surface adhesins, curli production is of particular importance for biofilm formation of *E. coli* due to their adhesive properties and role in attaching to eukaryotic cells (Gophna *et al.*, 2001; Kikuchi *et al.*, 2005). Curli fibers, which are rich in β-folded layers of amyloid fibers, are composed of a major (CsgA) and a minor (CsgB)

subunit (Saldana *et al.*, 2009). A combination of environmental stress and physiological change, such as temperature or osmolarity and slow growth rate, is likely associated with the induction of curli production (Barnhart and Chapman, 2006). A microarray study revealed that one strain of *E. coli* O157:H7 (EDL933) with strong biofilm formation showed a large amount of curli production, whereas a curli negative strain (86-24) failed to produce biofilms on abiotic surfaces (Lee *et al.*, 2011). Similarly, a positive effect on biofilm formation was observed with an *E. coli* mutant that overexperssed curli, whereas the ability to form biofilms was lost for an *E. coli* mutant that lacked curli production (Schembri et al., 2003; Vidal et al., 1998).

Two divergently transcribed operons, *csgBAC*-(*ymdA*) and *csgDEFG*, are found involved in curli production (Hammar *et al.*, 1995; Romling *et al.*, 1998; Uhlich *et al.*, 2001). Except *csgB* and *csgA* encoding two curli functional subunits, the rest genes that belong to the two operons regulate the assembly and secretion system of the two subunits of curli fibers (Loferer *et al.*, 1997). Curli expression strictly depend on *rpoS* gene through an complex transcription regulation for *csgD*, which encodes a transcription activator triggering the expression of *csgBAC*-(*ymdA*). It suggests that RpoS regulates *E. coli* biofilm formation in an indirect manner (Landini, 2009). In addition, it has been found that the second messenger (c-di-GMP) can interplay with *rpoS* and contributes to curli expression and cellulose biosynthesis (Landini, 2009; Weber et al., 2006).

3.3.3 Extracellular polysaccharide production

After attaching to a surface, bacterial cells produce increasing amounts of exopolysaccharides (EPS) that can harbor surrounding planktonic bacteria and provide architectures for biofilms. The 3-dimentioanl structured EPS not only allow biofilm to

keep its integrity, but also maintain bacterial proximity to ensure intimate cell-cell contact. Moreover, EPS allow creation of a microenvironment with accumulated nutrients and high water content that support bacteria growth. They also provide protective barriers by blocking the penetration of antimicrobial agents in biofilms (Flemming and Wingender, 2010). However, the composition and function of EPS on a molecular level has not been fully elucidated. Therefore, only several well-studied EPS molecules that have been shown to play important roles in biofilm formation will be discussed.

Cellulose is an abundant organic polymer in *E. coli* biofilm (Jonas *et al.*, 2007). In addition to curli production, *csgD* along with *rpoS* plays an important role in exopolysaccharide synthesis. Both cellulose and O-antigen polysaccharide production are found to be upregulated by *csgD* in *Salmonella* (Gibson et al., 2006; Latasa et al., 2005). More specifically, cellulose production is under regulation of a second messenger--AdrA protein, and a large cell-surface protein--BapA, both of which are activated by *csgD* gene (Landini, 2009; Zogaj et al., 2001). In fact, curli and cellulose usually form a honeycomb-like extracellular matrix that can assist *E. coli* adhesion and enhance bacterial resistance to dessication (Gualdi et al., 2008; Wang et al., 2006). This matrix also protects cells from harsh environmental conditions, such as desiccation, low osmolarity and low temperature, *et al*, due to its highly hydrophobic property. (Zogaj *et al.*, 2001).

Colanic acid, another major component of extracellular polysaccharide in *E. coli* biofilm, was initially identified from slimy colonies on favorable agar plates (Junkins and Doyle, 1992). It has been proposed that colanic acid production of *E. coli* O157:H7 is strain dependent. For strains with colanic acid production ability, the extracellular polysaccharide particles were found around the bacterial cells or on the glass surfaces at

the microcolony formation stage during biofilm formation (May and Okabe, 2008). Different from honeycomb-like structure formed by curli and cellulose, colanic acid is not important for bacterial adhesion, but critical for biofilm architecture development (Prigent-Combaret *et al.*, 2000). This exopolysaccharide alone can result in a thick flat biofilm structure, whereas it can connect with curli to form the mushroom-like structures in *E. coli* biofilm (May and Okabe, 2008).

Poly-N-acetyl-glucosamine (PNAG) was first described in *Staphylococcus aureus* and *S. epidermidis* as playing an important role in immune evasion and biofilm formation (Cramton *et al.*, 1999). The presence of PNAG in *E. coli* results in promoting attachment on abiotic surface, intercellular adherence, and biofilm formation (Wang *et al.*, 2004). By understanding the synthesis of PNAG, researchers found that glucose could induce PNAG production and resulted in enhanced production of biomass in *E. coli* biofilm (Cerca and Jefferson, 2008). Production of PNAG blocked the penetration of cationic surfactant cetylpyridinium chloride through the biofilm surface (Ganeshnarayan et al., 2009; Izano et al., 2008). Additionally, it has been found that PNAG is required for the binding of *E. coli* O157:H7 on alfalfa sprouts in aquatic environment (Matthysse *et al.*, 2008).

3.3.4 Quorum sensing

Quorum sensing is a cell-cell communication system regulated by stimulus and response relating to population density. When the stimulating molecules reach a concentration threshold, their specific responding receptors will be activated to induce certain gene expression. AHL (N-acyl-homoserine-lactone), AI-2 (autoinducer-2) and AI-3 (autoinducer-3) are quorum sensing molecules that have been identified to play important roles in bacteria biofilm formation.

Quorum sensing was first elucidated in the regulation of bioluminescence in *Vibrio fischeri* (Nealson *et al.*, 1970). AHL, regulated by LuxI in *V. fischeri*, binds to the LuxR protein and in turn activates the transcription of the luciferase operon for luminescence emission. SdiA, a LuxR homologue, has been identified as a receptor for AHLs molecules in *E. coli* and *Salmonella*, even these two species do not produce AHLs (Wang, 1991). AHLs molecules are sensed and bond with SdiA to promote *E. coli* biofilm formation and increase acid resistance. In contrast, indole, a signaling molecule accumulated in stationary phase, has been shown to repress to *E. coli* biofilm formation and acid resistance via the same quorum sensing system (Lee *et al.*, 2007). Thus, AHL and indole play opposite roles in regulation of *E. coli* biofilm formations.

The *luxS*/AI-2 system is another widespread quorum-sensing model that has been found in both Gram-positive and Gram-negative bacteria strains (Li *et al.*, 2007; Williams *et al.*, 2007). LuxS is an enzyme that catalyzes S-adenosyl-methionine (SAM) conversion to the precursor of AI-2. By identifying genes regulated by *luxS* with or without the presence of glucose, Wang and colleagues found that only a few genes were affected under both conditions. It suggested that LuxS was an important metabolic enzyme for *E. coli*, but the role of LuxS was conditionally dependent (Wang *et al.*, 2005). AI-2 molecules can pass through the outer membrane and be transported in *E. coli* and *Salmonella* cells by the periplasmic receptor LsrB (Walters and Sperandio, 2006). Barrios and colleague proposed that AI-2 could promote *E. coli* biofilm formation and alter biofilm structure via a motility quorum-sensing regulator MqsR. Their results showed that MqsR simulated flagellar motion and motility in *E. coli* by positively regulating the two-component motility regulatory system *qseBC* and flagellar related genes *flhD* and

fliA. With the presence of AI-2, the biomass and thickness of *E. coli* biofilm was promoted and the heterogeneity of biofilm structure was reduced (Barrios *et al.*, 2006).

Similar to AI-2, AI-3 has also been shown to be able to activate the quorum-sensing regulators, QseB and QseC, promoting flagella production, and subsequently resulting in biofilm formation (Sperandio et al., 2003; Sperandio et al., 2002). AI-3 is also associated with enterohaemorrhagic *E. coli* (EHEC) virulence by activating LEE and flagella genes. With the presence of AI-3, two divergent functional two-component systems, QseBC and QseEF are responsible for EHEc virulence developement (Walters and Sperandio, 2006). More specifically, QseBC system plays a role in transducing AI-3 signals towards activation of flagella regulon (Clarke *et al.*, 2006). QseEF system plays a role in transducing AI-3 signals to activate expression of the chromosomal LEE (Anand and Griffiths) pathogenicity island (Bjornsdottir et al., 2006; Walters and Sperandio, 2006). The LEE-encoded type III secretion system is activated and thus facilitates translocation of the virulence proteins to target epithelia cells (Bjornsdottir et al., 2006; Jarvis et al., 1995; Walters and Sperandio, 2006).

3.4 Pathogenic bacteria in multispecies biofilms *in vitro*

In multispecies biofilms, pathogenic bacteria can interact with adjacent bacteria by competitive and cooperative growth. To study the interactions between different bacteria species, dual-species biofilms are usually used to simulate natural biofilms in a simplified way. While this method does not replicate naturally formed biofilms, it has been instrumental in understanding the physiological and metabolic interactions between test bacteria cells.

3.4.1 Competition in biofilms

3.4.1.1 Co-existence of pathogens in biofilms

In dual-species biofilm studies, antagonistic interaction is used commonly to indicate a competitive growth between two bacteria species. For such studies, "antagonistic interaction" is considered the same as "competitive growth". The criteria used to determine whether an interaction is antagonistic is based on the quantitative results of total biomass or plate counts. When the total biomass or plate counts in dual-species biofilms is less than both of the individual monoculture biofilms, the interaction between the two bacteria species is considered to be antagonistic (Simoes *et al.*, 2007).

Antagonistic interactions have been found in dual-species biofilms formed by isolates from food processing environments. Similar results have been observed in dual-species biofilms formed by strains isolated from food processing environment and pathogenic bacteria. Sixteen out of 29 strains that were isolated from dairy and meat processing facilities showed negative impact on *L. monocytogenes* attaching on solid substances (Carpentier and Chassaing, 2004). A maximum 3-log colony-forming units (CFU) reduction of *L. monocytogenes* attaching on substrate was found with the presence of *Baccilus sp., Pseudomonas fluorescens* or an un-identified Gram-positive strain, respectively (Carpentier and Chassaing, 2004). This effect was consistently observed for a variety of materials, including stainless steel, polyurethane, and polyvinyl chloride conveyor belt that selected to simulate processing surfaces in meat premises (Midelet and Carpentier, 2002). These results indicated that pathogenic bacteria could co-exist with resident bacteria in processing environment, although competition took place between environmental strains and pathogenic cells.

3.4.1.2 Key factors affecting competition in biofilms

Competitive growth commonly occurs between native microflora associated with fresh produce and pathogenic bacteria. Several plant associated bacteria have been found to inhibit growth of pathogens on fresh produce. Two factors appear to be involved in the competitive growth, altered growth rates and antimicrobial agent production.

Theoretically, bacterial strains with faster growth rates will outcompete or inhibit the growth of others with slower growth rates in a nutrient limited environment. Referred to as the Jameson Effect, this results in a suppression of the maximum population density reached by the slower growing bacterium. In most cases, this competition happens when the strains require similar nutrients. For example, one enteric bacterium, *Enterobacter asburiae*, outcompeted *E. coli* O157:H7 on lettuce leaves by lowering nutrient accessibility of *E. coli* O157:H7 (Cooley *et al.*, 2006). However, no correlation has been found between growth rate and dual-species biofilms formation in static cultures (Simoes *et al.*, 2007). For example, a fast grower, *Klebsiella oxytoca*, was only a small portion of dual-species biofilms formed with a slow grower, *Burkholderia cepacia* (Komlos *et al.*, 2005). Moreover, intraspecies competition has been found between different bacterial serotypes in biofilms (Pan *et al.*, 2009). For instance, when serotypes 1/2a and 4b of *L. monocytogenes* were co-cultured to form dual-strain biofilms, 1/2a was more likely to dominate in mixed-culture biofilms due to its higher biofilm forming capacity in diluted medium, whereas a small number of the faster growing 4b serotype was in the biofilms (Pan *et al.*, 2010). Similar results were reported when different *E. coli* strains were used to form dual-species biofilms (Uhlich *et al.*, 2010). These results indicated that the

growth rate in planktonic culture might not predict the bacteria proportion in dual-species biofilms.

Antagonistic interactions might occur when a bacteriocin-producing bacterium is co-cultured with other susceptible strains. However, coexistence of bacteriocin-producing and bacteriocin-sensitive bacteria has been observed in biofilms communities. In general, antimicrobial compounds such as bacteriocin can result in killing or inhibition of taxonomic closely related species (Riley, 1998). Extensive surveys revealed that the production of antimicrobial agents in many bacteria isolates associated with plant tissue, such as *Pseudomonas fluorescens*, *P. aeruginosa*, *Bacillus* spp., *Aeromonas hydrophila*, inhibited *E. coli* O157:H7 growth on fresh produce (Liao and Fett, 2001; Schuenzel and Harrison, 2002). In biofilms, antagonistic compounds generated from producers mainly inhibit the proliferation of susceptible species, instead of killing all the competitors, to allow the producers to be numerically dominate in a complex community (Tait and Sutherland, 2002). Pihl *et al*. revealed that a *Staphylococcus epidermidis* biofilm could be replaced by the attachment and biofilm formation of *P. aeruginosa* on abiotic surfaces via competitive attachment and biofilm disruption. Further more, it had been proposed that the inhibited growth of *S. epidermidis* in biofilms was due to antistaphylococcal substance produced by *P. aeruginosa*. In fact, *S. epidermidis* cells were detached from the biofilm substrata rather than being killed by *P. aeruginosa*, which indicated that these two strains could coexist in aquatic environment (Pihl *et al.*, 2010).

3.4.2 Cooperation in biofilms

3.4.2.1 Increased growth of pathogens in biofilms

In contrast to competition, certain phytobacteria show positive effects on the growth of human pathogens, which is defined as cooperation. Studies have shown that there is heterotrophic cooperative growth between bacterial isolates recovered from food processing environments and pathogenic bacteria. For example, *Acinetobacter calcoaceticus*, isolated from a meat factory was able to increase the biovolume of *E. coli* O157:H7 in dual-species biofilm by 400-fold (Habimana, Heir, et al., 2010). Researchers evaluated the interaction between *E. coli* O157:H7 and biofilm-coated surfaces formed by resident bacteria strains isolated from a beef processing facility. Nearly all of the isolates were able to increase *E. coli* O157:H7 presence in dual-species biofilm compared to monoculture biofilm formed by *E. coli* O157:H7 (Marouani-Gadri *et al.*, 2009).

It has also been reported that intergeneric cooperative interaction could affect the distribution of pathogenic bacteria in dual-species biofilms. *E. coli* O157:H7 cells were gradually covered by a thick layer of *A. calcoaceticus* cells formed in biofilms under dynamic culture condition. Whereas, the reference *E. coli* O157:H7 strain does not adhere efficiently to solid surfaces and form biofilms, on its own, in continuous culture due to its deficiency of irreversible adhesion (Dewanti and Wong, 1995; Habimana, Heir, et al., 2010). When co-inoculated with *P. aeruginosa* under flow culture condition, *E. coli* O157:H7 showed a 100-fold increase in biovolume compared to initial attachment. However, this increase was not observed when *E. coli* was introduced to surface precolonized by *P. aeruginosa* biofilm under the same flow culture conditions. The authors concluded that habitat favorability was more critical than a preformed biofilm for pathogen proliferation (Klayman *et al.*, 2009). *L. monocytogenes* cells attached on solid substrates as single cells in monoculture biofilm, but the pattern changed when other

resident bacteria from dairy and meat industry were present (Carpentier and Chassaing, 2004). When co-colonized with *Kocuria varians*, *L. monocytogenes* cells accumulated around *K. varians* microcolonies. Alternatively, *L. monocytogenes* formed its own microcolonies when co-cultured with *Comamonas testosteroni* (Carpentier and Chassaing, 2004).

3.4.2.2 Key factors affecting cooperation in biofilms

While it has been accepted that the native microflora of plants have antagonistic interactions with human pathogens in biofilms, only a limited number of studies have addressed mechanisms for cooperative growth in dual-species biofilms. Studies that have addressed cooperative growth in such biofilms measured factors such as co-colonization, co-aggregation, growth rates, and EPS production.

It has been reported that plant related bacteria promoted the growth of pathogens by increasing nutrient availability on vegetable tissue. For example, the survival of *E. coli* O157:H7 was promoted by *Wausteria paucula*, a common epiphytic bacteria, in the rhizosphere and leaf-surface of lettuce after co-inoculated on lettuce seed. It was suggested that the interaction between the two strains might due to the modification of plant tissue by *W. paucula*, which provided suitable niches for *E. coli* O157:H7 (Cooley *et al.*, 2006). This hypothesis was supported by a study focusing on interaction between *Dickeya dadantii* and *E. coli* O157:H7 on lettuce leaves (Yamazaki *et al.*, 2011). Pectate lyases secreted by the phytopathogen *D. dadantii* caused the maceration of plant tissue and further enhanced *E. coli* O157:H7 proliferation on the lettuce leaves (Yamazaki *et al.*, 2011).

When aggregation occurs among heterogeneous microorganisms, the process is defined as co-aggregation. Co-aggregation interaction is considered to contribute to naturally occurring biofilms in two ways. Firstly, developing biofilms can attract bacteria cells in suspension by specific cell-cell bonding and consequently cause adhesion. Secondly, different bacterial species can co-aggregate into clumps in suspension and further adhere on abiotic surfaces to build up biofilms (Rickard *et al.*, 2003). At this stage, some attached cells transit from transient attachment to permanent attachment. The specificity of co-aggregation is mediated by the specific bonding between adhesins and related receptors on different cells (Rickard *et al.*, 2003).

Selected factors have been studied to elucidate mechanisms of cooperative growth in dual-species biofilms. Bacterial growth rate and exopolysaccharide production showed no direct correlation with its numerically proportion in dual-species biofilm formation (Carpentier and Chassaing, 2004; Pan et al., 2009; Simoes et al., 2007). Researchers hypothesize that synergistic interaction in dual-species biofilms might be associated with distinctly different physiological and metabolic properties between specific strains that may compensate with each other (Andersson et al., 2008; Burmolle et al., 2006; Simoes et al., 2007). However, more studies are required to better understand the mechanism of synergistic interaction.

3.5 Potential food safety issues related to biofilms in fresh-cut processing facilities

3.5.1 Biofilms as a protective environment for pathogens

The multispecies biofilm enhances pathogens' resistance to disinfectants, increasing its likelihood of survival in food processing environment. In dual-species biofilms, *E. coli* O157:H7 retention was enhanced when facing aggressive washes

(votexing) and 5% H_2O_2 treatment (Uhlich *et al.*, 2010). Similarly, co-cultured biofilms composed of *L. monocytogenes* and *Lactobacillus plantarum* showed increased resistances to benzalkonium chloride and peracetic acid (Van der Veen and Abee, 2011). In general, bacterial resistance to disinfectants and sanitizers is affected by the biodiversity and interspecies relationships in biofilm community (Berry *et al.*, 2006). The enhanced resistance may diminish the efficacy of current sanitizing techniques on inactivating opportunistic pathogen cells embedded in biofilms. Thus, pathogenic bacteria may survive and persist in food processing environment via a specific partnership with other bacteria in produce processing environment. It has been hypothesized that the partner strain is most likely to be resident bacteria that commonly present in a food processing facility.

Continuous exposure to a disinfectant may impact microbial spatial arrangement in biofilms. For example, multispecies biofilms produced by *Kocuria* spp., *Brevibacterium linens*, and *Staphylococcus sciuri* under exposure of free chlorine showed a special distribution where *Kocuria* spp. cells formed microcolonies surrounded by the rest two tested bacteria. This spatial arrangement disappeared when the free chlorine was absent (Leriche *et al.*, 2003). These results suggest that certain bacteria are potentially protected in biofilms depending on microbial interactions with the presence of disinfectants.

3.5.2 Biofilm as a source of cross-contamination

Current disinfection procedures were based on results measured in terms of effects on planktonic bacteria. These procedures may not be sufficient to mitigate biofilm growth in processing facilities. For example, two-log units reduction of bacterial numbers

was achieved in multispecies biofilms formed by *Listeria monocytogenes*, *Pseudomonas fragi* and *Staphylococcus xylosus*, when the biofilms were exposed to 1000 ppm free chlorine. However, planktonic culture of all three organisms were completely eliminated in 10 ppm free chlorine (Norwood and Gilmour, 2000). Consequently, it is difficult to completely remove or kill protected bacterial cells by using daily cleaning and disinfection operations. Therefore, such incomplete removal of biofilms can be a food safety concern due to the protective role for bacterial pathogens.

Biofilms not killed by disinfectants can be a source of contamination of fresh-cut products in processing facilities. The two primary mechanisms of cross-contamination are biofilm-food direct contact and wash water. More than 3 log CFU/cm^2 *E. coli* O157:H7 were transferred from biofilm to fresh produce after 30 min of contact. Transference was also observed between *E. coli* O157:H7 biofilm and raw meat or deli meat products (Silagyi *et al.*, 2009). There are two ways for embedded bacteria switch to planktonic living status and result in cross-contamination via wash water. A strong shear force, such as water turbulence, which applied to a surface can rip off biofilm pieces including pathogens that are normally embedded in biofilms (Maukonen *et al.*, 2003). Alternatively, pathogens can be released back to the environment when a matured biofilm forms (Ma *et al.*, 2009). Detached bacteria, potentially including human pathogens, become planktonic and float around processing facilities in wash water. Eventually, they may reattach to another abiotic surface and start a biofilm lifecycle or attach to food surfaces (Gil *et al.*, 2009).

To reduce potential risk of contamination caused by biofilms, it is benefical to understand the role of bioflms on the survival of foodborne human pathogens in food

processing facilities. Although many studies have been conducted in meat and dairy facilities, few studies have examined biofilms in fresh produce facilities.

Chapter 4: Native microflora in fresh-cut processing plants and their potentials of biofilm formation

Adapted from: Liu, N.T., Lefcourt, A.M., Nou, X., Shelton, D.R., Zhang, G., Lo, Y.M., 2013. Native Microflora in Fresh-Cut Produce processing plants and Their Potentials for Biofilm Formation. J Food Protect. (5) 827-832.

4.1 Introduction

With the lack of practical kill steps, fresh-cut produce processors continue to rely on sanitizing washes to maintain the microbiological quality of fresh-cut products and to prevent potential cross contaminations by foodborne pathogens. Rigorous sanitization of the processing environment and equipments is also crucial for preventing transference of pathogenic microorganisms to processed produce (Gibson *et al.*, 1999a; Gil *et al.*, 2009). Microbial communities in fresh produce processing facilities are composed of diverse microbial species. They may include bacteria proficient at forming biofilms, which can potentially protect food spoilage and pathogenic bacteria to survive in the processing plants (Bridier, Briandet, et al., 2011; Gibson et al., 1999a). Under certain conditions microorganisms in biofilms can detach and disperse, leading to potential contamination of food products (Midelet and Carpentier, 2002; Silagyi *et al.*, 2009).

Several studies have been conducted to examine biofilm formation and its association with the contamination of a variety of foods. It has been documented that food contaminations due to contacting with biofilms can occur in dairy, meat, and fruit juice processing plants (Midelet and Carpentier, 2004; Pérez-Rodríguez *et al.*, 2008). In dairy and meat processing lines, biofilms are often found at locations that are less accessible for routine sanitization (Chmielewski and Frank, 2003). These biofilms may increase the likelihood of the presence of pathogenic bacteria in the plant (Chmielewski

and Frank, 2003). It was suggested as early as 1998 that foodborne outbreaks associated with fresh-cut produce might be related to the existence of biofilms in fresh-cut processing facilities (Carmichael *et al.*, 1998). However, no direct evidence of such a link has been established.

The temperature in fresh-cut processing plants must be maintained at 4 ^{o}C or below to be in compliance with FDA regulations (FDA, 2008). In reality, the temperature may fluctuate in the processing environment and temperature may occasionally rise for other reasons. Microenvironments with elevated temperature may also exist in the processing plants. This environment, combined with the high humidity, is vulnerable to the establishment of biofilms by native microflora that survive routine sanitization. Such microbial communities can be consisted of epiphytic, soil-associated, human pathogenic and other bacteria from various environmental sources (Gil et al., 2009; Harris et al., 2003). Some of these bacteria have been shown capable of forming biofilms on produce surfaces in laboratory experiments (Rudrappa *et al.*, 2008), suggesting that they could play a role in forming biofilms on processing surfaces in the facilities. Resident strains from various surfaces in a raw vegetable processing facility were shown to have a higher biofilm forming capacity than a laboratory *E. coli* strain (Van Houdt *et al.*, 2004). A large variation in biofilm forming capacity among the isolates was observed in this study; however, the composition of the bacterial community was not fully determined by species identification (Van Houdt *et al.*, 2004).

To better understand the potential of biofilm formation by native microflora from fresh-cut processing plants, samples were obtained from multiple locations in two local

facilities. All samplings were conducted after routine daily sanitization. Biofilm formation potentials of bacteria isolated from the samples were evaluated.

4.2 Materials and Methods

4.2.1 Sampling at fresh-cut processing plants

Two mid-sized local fresh-cut produce processing plants, which processed a variety of fresh produce including leafy greens and fruits, were sampled for native microflora at multiple food contact and non-contact surfaces. Samples were taken from corresponding locations in both plants during plant visits in the fall of 2010. All samples were taken after routine daily cleaning and disinfection of the facilities and before the start of the morning shift. An area of 100 cm^2 of the selected surface was defined using a sterile template and sampled by vigorously rubbing 5 times using a sterile sponge (Whirl-pak, Nasco, Fort Atkinson, WI) hydrated with 15 ml sterile phosphate buffered saline (PBS, pH 7.2) (Fisher Scientific, Pittsburg, PA). For surfaces with non-flat contour, the sampled areas were approximated. To neutralize the residual chlorine from facilities surfaces after sanitization, PBS was supplemented with chlorine neutralizing reagent followed the manufacture instruction (Permachem reagent, Hach, Düsseldorf, Germany). The materials of sampled surfaces included Acrylic-modified cement floor (AMCF), high-density polyethylene (HDPE), nylon (NL), polyvinyl chloride (PVC), and stainless steel (SS). Sample sponges were placed in stomacher bags (Whirl-pak, Nasco, Fort Atkinson, WI), and transported to the laboratory on ice. Samples were processed within 5 h.

4.2.2 Recovery of bacterial strains

Sample sponges were pummeled for 2 min in a stomacher (Seward, Lab Blender, Batavia, IL) and the liquid squeezed out by hand. Each sample was ten-fold serially diluted and then spiral plated on two TSA (Tryptic Soy Agar, BD, Franklin Lakes, NJ), one MAC (MacConkey, Neogen, Lansing, MI) and one B-medium (Kato *et al.*, 2001) plates. MAC plates and one set of TSA plates were incubated at 30 °C for 2 days. B-medium plates and the other set of TSA plates were incubated at 10 °C for 10 days. TSA plates incubated at 30 ^{o}C were used to determine mesophilic bacteria counts and MAC plates were used for the recovery of Gram-negative bacteria. The population of psychrotrophic microflora was determined using TSA and B-medium plates incubated at 10 ^{o}C.

Aliquots of all samples collected from processing plants were also incubated in low nutrient liquid medium with the presence of stainless steel beads to allow formation of heterogeneous biofims. A 100 µl aliquot of the sample was inoculated into 10 ml broth supplemented M9 medium (BD, Franklin Lakes, NJ) (M9 salts supplemented with 0.5% glucose) and 5% LB broth (BD, Franklin Lakes, NJ) (Castonguay *et al.*, 2006) in a 50 ml tube containing 4 Type 316 stainless steel (SS) beads (d=6.3 mm) (Biosurface Technology Corp., Bozeman, MT) and incubated at 10 ^{o}C for 10 days with moderate shaking. After incubation, the SS beads were rinsed with sterile water to remove unattached and weakly attached bacterial cells. Then the beads were immerged in 10 ml sterile PBS and vigorously vortexed for 1 min. Recovered bacterial cells were spiral plated, incubated, and enumerated using the same procedures described above.

4.2.3 Screening isolates for biofilm formation capacity

Single bacterial colonies with distinct morphology (up to 3 colonies for each type) on the recovery plates were inoculated into 96-well microtitre plates containing 200 µl of 1:10 dilution of TSB (Tryptic Soy Broth, BD, Franklin Lakes, NJ) and simultaneously streaked on TSA plate. Inoculated microtitre plates were incubated at 30 °C for 24 h to examine the biofilm forming potential of each colony using the crystal violet staining method described by Stepanović (Stepanović *et al.*, 2000). Total biomass formed by each colony was estimated based on optical density at 590 nm (OD_{590}) measurements as described by Stepanović (Stepanović *et al.*, 2000). Colonies with OD values that exceeded a threshold (OD_c), calculated as the sum of the average OD value for the negative controls plus three times the standard deviation, were considered being biofilm formers. Specifically, the potential of biofilm formation for each isolate was categorized as strong ($OD_{590} > 4\ OD_c$), moderate ($2\ OD_c \leq OD_{590} < 4\ OD_c$), weak ($OD_c \leq OD_{590} < 2\ OD_c$), or non-biofilm former ($OD_{590} < OD_c$) based on the total biomass measured as optical density. For each sample, isolates with similar colony morphology and biofilm formation potentials were considered identical and one representative was further streaked on TSA plates for single colonies. All purified isolates were stored at -80 °C.

4.2.4 Species identification and verification of biofilm forming capacity of representative isolates

Purified isolates were subjected to species identification using the Biolog Gen III Microbial ID system (Biolog, Farmingdale, NY) following the manufacturer's instructions. For all identified isolates of a certain species, one isolate was selected to further evaluate biofilm forming capacity. Each selected isolate was incubated overnight

to reach stationary phase in TSB (BD, Franklin Lakes, NJ) at 30 °C with 150 rpm/min orbital shaking. The cultures were centrifuged at 4,000 ×g at 10 °C for 5 min and the resulting pellet washed three times with sterile PBS. Pellets were resuspended in sterile PBS and cell density adjusted to approximately 1.0 OD_{600} unit, which approximately equals to 9.0 log CFU/ml. The resuspended cell cultures were ten-fold serially diluted in a 1:10 dilution of TSB to achieve a targeted inoculum concentration of 3.0 log CFU/ml. Two hundred microliter of each inoculum was added to each well on a 96-well microtitre plates with 6 replicates. The plates were incubated at 30 °C with 80 rpm/min orbital shaking for 24 h to evaluate biofilm formation. The measurement of total biomass was carried out by crystal violet staining method as previously described.

4.3 Results

4.3.1 Recovery of mesophilic and psychrotropic bacteria from fresh-cut processing plants

In the present study, a variety of food contact and non-food contact surfaces in two local fresh-cut processing plants were sampled after routine daily sanitization and the microflora were characterized. Table 4.1 shows the aerobic microbial populations on sampled surfaces expressed as the average for the two plants. Viable bacterial cells were not detected or detected at very low levels on most food contact surfaces. Bacterial populations on the carrot/potato peeler brushes, containers for washing purposes and the blades of cutting knives were approximately 2.0 log CFU/cm^2. In contrast, higher bacterial counts (up to 5.8 log CFU/cm^2) were obtained from most non-food contact surfaces, including the conveyor frames, knife handles, and the floor (Table 4.1). In an attempt to increase the recovery of bacteria with strong potential of biofilm forming

capabilities, samples from the processing plants were also incubated with stainless steel beads in a low nutrient broth. It was speculated that bacterial cells with stronger biofilm forming potentials would be more likely to attach to this solid substrate and form biofilms. After incubation, the SS beads were rinsed and bacterial cells attached to the beads were then released and plated on non-selective bacterial media and incubated at 10 or 30 ^{o}C (Table 4.2). Bacterial cells were recovered from the SS beads for samples collected from multiple locations, including both food contact and non-food contact surfaces. The diverse colony morphology suggested the formation of heterogenous biofilms on the SS bead during the incubation period at 10 ^{o}C.

Table 4.1 Aerobic plate counts (log CFU/cm^2) of native microflora from local fresh-cut processing plants by direct spiral plating on TSA, B-medium and MAC plates [a].

	Sample Locations	*10 °C* [b]		*30 °C* [b]	
		TSA	*B-medium*	*TSA*	*MAC*
Food	Peeler brush (NL) [c]	5.62 [d]	5.77	5.85	5.53
contact	Container (HDPE)	4.62	3.57	3.95	4.24
surfaces	Conveyor belt (HDPE)	ND	ND	ND	ND
	Cutting broad (HDPE)	ND	ND	ND	ND
	Knife blade (SS)	5.42	5.51	4.92	4.92
	Slicer (SS)	ND	ND	ND	ND
Non-food	Conveyor belt (HDPE)	4.04	4.41	ND	ND
contact	Conveyor frames (SS)	5.06	5.24	4.88	4.84
surfaces	Knife handle (PVC)	4.80	4.74	3.50	3.20
	Floor (AMCF)	3.99	3.36	3.48	2.64
	Stainless steel table (SS)	3.91	3.33	ND	ND

[a] Data represents the average of the two sampled local plants and the variability between the two samples was low.

[b] Incubation temperature used for spiral plated samples.

[c] Letters in parentheses indicates the material of the surface the sample was taken.

[d] ND, not detected at detection limit of 1.0 log CFU/cm^2.

Table 4.2 Aerobic plate counts (log CFU/cm^2) for samples from biofilms formed on SS beads at 10 ^{o}C by spiral plating on TSA, B-medium and MAC plates [a].

	Sample Locations	*10 ^{o}C* [b]		*30 ^{o}C* [b]	
		TSA	*B-medium*	*TSA*	*MAC*
Food	Peeler brush (NL) [c]	1.93	2.13	2.31	1.52
contact	Container (HDPE)	ND[d]	1.86	ND	ND
surfaces	Conveyor belt (HDPE)	ND	ND	0.51	ND
	Cutting broad (HDPE)	ND	ND	ND	ND
	Knife blade (SS)	1.80	2.05	2.07	ND
	Slicer (SS)	ND	ND	ND	ND
Non-food	Conveyor belt (HDPE)	ND	ND	ND	ND
contact	Conveyor frames (SS)	4.21	3.51	2.73	ND
surfaces	Knife handle (PVC)	1.35	1.53	2.16	1.80
	Floor (AMCF)	5.83	5.39	5.11	3.93
	Stainless steel table (SS)	ND	ND	ND	ND

[a] Raw samples from all locations in Table 1 were tested and the results were the average of the two local plants and the variability between the two samples was low.

[b] Recovery temperature used for recovery of samples released from SS beads.

[c] Letters in parentheses indicates the material of the surface the sample was taken.

[d] All numbers denote the number of colonies (log CFU/cm^2) isolated from SS beads after enrichment. The numbers can be an indication of biofilm formation on SS beads but do not correlate with the numbers of bacterial in the original samples. ND: not detected at detection limit of 1.0 log CFU/cm^2.

4.3.2 Screening for the potentials of biofilm formation

Selected single colonies obtained by directly plating samples from the processing plants (Table 4.1) and by plating bacterial cells bound to SS beads after incubation (Table 4.2) were tested for their biofilm formation potentials using crystal violet staining (Stepanović *et al.*, 2000). Representative distributions consisting of four types of biofilm formers were showed as percentile in Figure 4.1. A three-way Chi-square test (SPSS 17.0, IBM, Armonk, NY) was used to assess the recovery method and sampling surface on the distribution of the isolates relative to their ability of biofilm formation on microplates. The methods used to recover the isolates significantly ($p \leq 0.05$) affected this distribution. The distribution significantly shifted toward strong biofilm formers when the isolates where recovered from the SS beads. After recovery from SS beads, the difference in distribution was statistically significant ($p \leq 0.05$) for the isolates recovered from food contact and non-food contact surfaces. However, the effect of the surface type on the distribution was less pronounced than the methods used for obtaining the isolates.

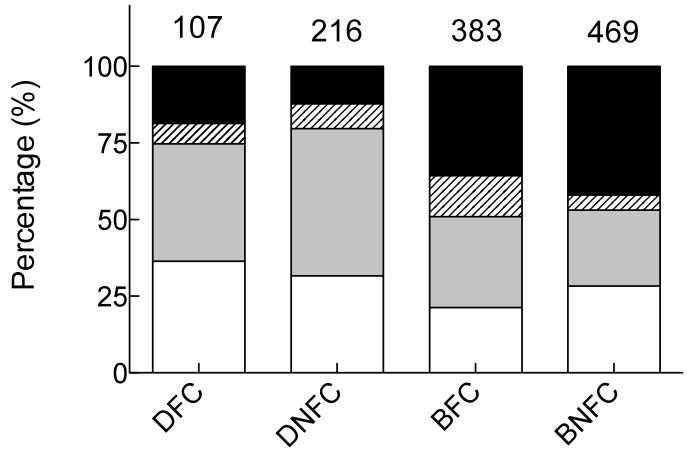


Figure 4.1 Distribution of bacterial isolates relative to ability of biofilm formation in microplates. Stacked bars represent the percentage of the isolates in each category. Number on top of each column was the amount of tested isolates. ☐ Non-biofilm formation; ▭ Weak biofilm formation; ▨ Moderate biofilm formation; and ■ Strong biofilm formation. Numbers over the stacked bars indicates total isolates tested. Letters under the stacked bars indicate method of recovery and the type of surfaces for the isolates. DFC: Direct plating, from food contact surfaces; DNFC: Direct plating, from non-food contact surfaces; BFC: SS beads, from food contact surfaces; and BNFC: SS beads, from non-food contact surfaces.

4.3.3 Species identification

Representative isolates from different type of samples and with different colony morphology were selected for species identification using Biolog GenIII microbiological ID system. The 117 selected isolates were identified as belonging to 23 genera with 105 belonging to 28 species, and 12 belonging to *Enterobacter, Pseudomonas, Arthrobacterim, Microbacterium,* and *Curtobacterium* genera without species identification (Table 4.3). Gram-negative species were abundantly represented among isolates from various surfaces. *Pseudomonas fluorescens*, *Rahnella aquatilis,* and *Ralstonia insidiosa* were the most abundant among the isolated strains. They were isolated from multiple food contact and non-food contact surfaces using either directly plating or plating following biofilm formation on SS beads.

4.3.4 Evaluation of biofilm-forming capacities of selected isolates

Among the strains tested for biofilm forming potential, *Burkholderia caryophilli*, *Klebsiella pneumoniae*, and *Ralstonia insidiosa* strains exhibited strong capacity of biofilm formation on microtitre plates, as judged by crystal violet staining of attached biomass. *Flavimonas orizyhabitans*, *Flavobacterium resinovorum, Pseudomonas fluorescens*, *Sphingomonas terrae*, *Stenotrophmonas rhizophila*, and *Raoutella planticolla* also displayed moderate biofilm formation. All other tested strains displayed low to no biofilm formation on the microtitre plates.

Table 4.3 Species identification and biofilm formation potentials of bacterial isolates collected from two local fresh-cut processing plants

Species	Potential Source[a]	Surface Type[b]	Surface Material[c]	Biofilm Formation[d]	Isolates Number
Acinetobacter genospecies	Soil	F	NL	+	3
Arthrobacter spp.	Plant*	NF	PVC	0	2
Bacillus pumilus	Rhizosphere	F, NF	HDPE, PVC	+	7
Brevibacterium frigoritolerans	Soil	F	HDPE	0	1
Burkholderia caryophylli	Plant*	NF	AMCF	+++	1
Burkholderia cepacia	Animal*	NF	AMCF	++	1
Chryseobacterium indoltheticum	Plant	F	NL	+	2
Corynebacterium bovis	Plant*	F	NL	0	1
Curtobacterium spp.	Plant	F, NF	HDPE, AMCF	0	3
Enterobacter cloacae	Animal, soil, plant	NF	AMCF, NL, SS	0	6
Enterobacter kobei	Animal, , soil, plant	NF	AMCF	+	1
Enterobacter spp.	Animal, soil, plant	F	NL	+	1
Flavimonas oryzihabitans	Animal*	F	HDPE	++	1
Flavobacterium resinovorum	Soil, water, plant	NF	AMCF	++	1

Klebsiella pneumoniae	Animal, plant	F	NL	+++	3
Microbacterium spp. *(CDC.A-5)*	Soil	F	NL	0	1
Paenibacillus amylolyticus	Water, rhizosphere	F, NF	HDPE, PVC, SS	0	3
Paenibacillus pabuli	Water, rhizosphere	F, NF	PVC	0	4
Pectobacterium cypripedii	Plant*	F	SS	0	1
Pseudomonas fluorescens	Plant	F, NF	HDPE, NL, PVC, SS	++	15
Pseudomonas maculicola	Plant*	F	NL	+	1
Pseudomonas marginalis	Soil, plant	F	NL	+	1
Pseudomonas tolaasii	Plant	F	NL	+	1
Pseudomonas spp.	Plant	F	NL	+	5
Rahnella aquatilis	Rhizosphere, water	F, NF	NL, SS	+	17
Ralstonia insidiosa	Animal	F, NF	HDPE, PVC, SS	+++	14
Raoultella planticola	Animal*	NF	AMCF, SS	++	4
Rhizobium radiobacter	Plant*	F, NF	PVC, SS	+	4
Staphylococcus epidermidis	Animal	F, NF	PVC, SS	+	2
Staphylococcus kloosii	Animal	NF	PVC	+	2
Staphylococcus xylosus	Animal	F, NF	SS, PVC	+	4

Sphingomonas terrae	Soil, plant	NF	SS	++	1
Stenotrophomonas rhizophila	Plant	F	NL	++	3

[a] Environments where the bacterial species is most frequently isolated in the literature. Symbol * denote plant or human pathogen, including opportunistic pathogen.

[b] Surface type for the sample. F: Food contact surface, NF: Non-food contact surface.

[c] The materials of sampled surfaces: AMCF, Acrylic-modified cement floor; HDPE, high-density polyethylene; NL, nylon; PVC, polyvinyl chloride; and SS, stainless steel.

[d] Biofilm formation capacity of one representative isolate. +++, ++, +, and 0 represent strong, moderate, weak, and no biofilm formers.

4.4 Discussion

In the present study, two local fresh-cut processing plants were sampled after routine daily sanitization and the native microflora were characterized. Although data from fresh-cut processing plants is limited, previous studies have examined the microflora in dairy, meat and fish facilities (Bagge-Ravn *et al.*, 2003; Bore and Langsrud, 2005; Marouani-Gadri *et al.*, 2009). The aerobic bacteria counts in those studies reached up to 10^4-10^5 CFU/cm^2 on sampled surfaces after cleaning and disinfection in the plants. The aerobic bacteria counts in the two fresh-cut processing plants sampled in this study ranged from undetectable to around 10^5 CFU/cm^2 after daily sanitization. Besides the routine sanitization, the lower operating temperature was used to inhibit bacteria growth in fresh-cut processing plants and the meat and dairy plants. The bacterial density determined in this study was in the same range as the microflora in meat processing lines. Kaneko and colleagues reported that the interior surfaces of washing, slicing, dewatering and blending equipments, the slicer, and the floor surfaces remained high, with aerobic plate counts around 10^5 CFU/cm^2 even after cleaning and disinfection in some ready-to-eat fresh vegetable processing environments (Kaneko *et al.*, 1999). In contrast, in this study only floors were found with counts around 10^5 CFU/cm^2 after cleaning and disinfection, indicating the microflora in the fresh-cut processing plants vastly differ depending on the practices in the facilities. In the two produce processing plants studied, bacterial counts on several food contact surfaces were below the detection limit. These locations included the conveyor belts, the cutting broads and the slicers. Similar results for cutting boards were also found in Kaneko's study. Among all sampled locations, no particular material was found to significantly differ from the others in terms of remaining

bacteria density after sanitization. Similar conclusion was reached in another study carried out by Marouani-Gadri in a beef-processing plant (Marouani-Gadri *et al.*, 2009).

To our knowledge, only a few studies have characterized the native microflora in fresh-cut processing plants. Van Houdt and colleagues investigated the quorum sensing molecule production of isolates collected from the native microflora in vegetable processing plants. However, only a limited number of isolates were subjected to species identification, which included *Vibrio diazotrophicus*, *Serratia plymuthica* and *Panthoea agglomerans* (Van Houdt *et al.*, 2004). Kaneko and colleague also examined the bacterial composition of the microflora in two ready-to-eat fresh vegetable processing plants and reported the presence of *Escherichia coli* and *Bacillus cereus* (Kaneko *et al.*, 1999). In this study, bacterial strains belonging to 28 species were isolated, including soil bacteria, plant related bacteria, coliforms, and opportunistic plant or human pathogenic bacteria. There was a significant overlapping of microbial communities between our findings and those from previous studies of meat and dairy processing facilities (Bagge-Ravn et al., 2003; Ellerbroek, 1997; Marouani-Gadri et al., 2009; Mettler and Carpentier, 1998). *Pesudomonas* was frequently isolated in both fresh produce and meat/dairy processing environments. Other genera, including *Staphylococcus*, *Bacillus*, *Corynebacterium*, *Enterobacter*, *Flavobacterium*, and *Klebsiella*, were commonly present in various agricultural product processing facilities. However, several plant and soil related bacteria, including *Rhanella*, *Ralstonia,* and *Rizobium,* were isolated with high frequencies from produce processing environments and seemed major components of native microflora in such places.

Raw produce sources, workers hygiene practices, sanitization practices and environmental factors all can affect the composition of microflora in particular areas (Pérez-Rodríguez *et al*., 2008). Therefore, the microbial community composition is highly dynamic. While the presence of many of the species in the facilities could be transient, it is likely that some species are adapted to the processing facilities environment and have become part of the resident microflora.

The presence of biofilms in food processing facilities may be related to foodborne outbreaks (Heaton and Jones, 2008). Once a biofilm is formed, it can enhance the resistance of microorganisms by creating a protective structure around bacteria, which in turn reduces the efficacy of cleaning and disinfection operations (Gibson *et al.*, 1999a). Greater survival rate of *E. coli* O157:H7 was observed in mixed culture biofilms than its monoculture, when biofilms were treated with 5% H_2O_2 (Uhlich *et al.*, 2010). Considering the potential for pathogens to transfer from biofilms to processed food surfaces by direct contact or physical forces applied during food production (Pérez-Rodríguez *et al.*, 2008), it is critical to control the presence of biofilms in fresh cut processing facilities. In a previous study, only two species with relatively strong biofilm forming capacities, *Serratia plymuthica* and *Panthoea agglomerans,* were isolated from a raw vegetable processing line (Van Houdt *et al.*, 2004). In this study, we isolated multiple bacterial species with strong biofilm forming potentials (Table 3). Several strong and moderate biofilm forming bacterial species were recovered after allowing biofilm formation on SS beads at 10 ^{o}C, suggesting a high possibility of biofim formation in produce processing facilities by the resident microflora at low temperature.

In particular, the frequent isolation of *Pseudomonas fluorescens* and *Ralstonia insidiosa* from various surfaces suggested they were established residents in the sampled plants. Examination of interactions of these bacteria with foodborne bacterial pathogens could provide insights on the survival of the pathogenic bacteria in food processing environments.

4.5 Conclusion

The microbial community in two mid-sized fresh-cut produce facilites was composed of over 30 bacterial species that belonged to 23 genera, including phytobacteria, soil related bacteria and opportunistic human pathogens. The most commonly identified bacteria species included *Pseudomonas fluorescens*, *Rahnella aquatilis* and *Ralstonia insidiosa*. Of note, *P. fluorescens* and *R. insidiosa* were moderate and strong biofilm producer, suggesting that these two species were established residents in the sampled facilites. Among all recovered isolates, approximately 30% were strong or moderate biofilm producers, suggesting a high possibility that native microflora produced biofilms in processing facilities.

4.6 Acknowledgements

The author thanks the two undisclosed fresh-cut processing plants for granting us access to their facility for this study.

Chapter 5: Dual-species biofilms formed by *Escherichia coli* O157:H7 and environmental bacteria isolated from fresh-cut processing plants

5.1 Introduction

In recent years, fresh produce has been increasingly recognized as an important vector for the transmission of foodborne pathogens, including enterohemorrhagic *Escherichia coli*, *Salmonella enterica* and *Listeria monocytogenes,* and has been implicated in a rising number of wide spread foodborne outbreaks (CDC, 2011b). Cross-contamination during post-harvest processing is thought to play a major role in the process of pathogen transmission (Allende *et al.*, 2004). In fresh produce processing plants, cross-contamination can involve wash-water-mediated transfer or direct contact with a contaminated surface (Delaquis *et al.*, 2007). It has been suggested that biofilms might facilitate cross-contamination by harboring pathogens and thus protecting them from cleaning and sanitation operations (Pérez-Rodríguez *et al.*, 2008).

Particularly, multi-species biofilms formed in food processing plants were suspected of being sources of cross-contamination in previous reports (Hansen *et al.*, 2007; Nadell *et al.*, 2009). For example, in meat and dairy processing plant, *L. monocytogenes* was isolated from surfaces where biofilms were expected to have formed by native microflora (Chmielewski and Frank, 2003). Several bacteria strains isolated from meat processing plants, including *Acinetobacter calcoaceticus* and *Pantoea agglomerans*, were characterized as "co-colonizers" that could promote weak or non-biofilm producers to attach to solid substrate and consequently form biofilms (Habimana, Heir, et al., 2010; Habimana, Moretro, et al., 2010). Furthermore, dual-species biofilms formed by *L. monocytogenes* and *Lactobacillus plantarum* have been reported to exhibit enhanced resistance to disinfection treatments in comparison to monoculture biofilms

formed by either species (Van der Veen and Abee, 2011). These findings suggested that biofilms could enhance the survival of bacterial pathogens in food processing plants.

In contrast, biofilm formation by resident microflora in fresh produce processing facilities has received limited attention. Van Houdt and colleagues previously reported that most of the bacterial isolates from equipment surfaces in raw vegetable processing plants could form biofilms (Van Houdt *et al.*, 2004). Recently, we reported the frequent isolation of several bacterial species with strong biofilm formation potentials from food contact and non-food contact surfaces in fresh-cut processing plants (Liu *et al.*, 2013). However, biofilms formation by native microflora in fresh-cut processing plants as a risk factor by potentially harboring foodborne pathogens has not been clearly established. In this study, we examined the dual-species biofilm formation by selected environmental bacterial strains isolated from fresh-cut processing plants and *E. coli* O157:H7, which has been associated with severe produce outbreaks (Critzer and Doyle, 2010; Uhlich et al., 2008).

5.2 Materials and Methods

5.2.1 Environmental bacteria and *Escherichia coli* O157:H7 strains

In the previous study (Chapter 4), multiple bacterial strains with various biofilm forming capacities were isolated from samples collected at two fresh-cut processing facilities. Species identification using Biolog Gen III Microbial ID system (Biolog, Hayward, CA) placed these isolates into 13 Gram negative bacterial species (Liu *et al.*, 2013). For this study, one strain from each of the 13 species was randomly selected for testing (Table 5.1).

Table 5.2 listed all *E. coli* O157:H7 strains used in this study. FS4052 is a derivative of the non-pathogenic *E. coli* O157:H7 strain ATCC 43888 (FS4108) harboring a plasmid expressing the green fluorescence protein (GFP) (Fratamico *et al.*, 1997). Other *E. coli* O157:H7 strains were isolates from infected animal or human, or from tainted fresh produce associated with foodborne outbreaks. Curli production by the *E. coli* O157:H7 strains were determined using Congo Red indicator (CRI) agar plates (Carter *et al.*, 2011). After incubating on CRI agar at 28 ^{o}C for 2-3 days, *E. coli* O157:H7 strains with dark red colonies were categorized as positive (+) and those with white colonies as negative (-) for curli production.

Each of the environmental isolates in Table 5.1 was evaluated for dual-species formation with *E. coli* O157:H7 strain FS4052 (Table 5.2). Further, *R. insidiosa* strain FC1138 was used to evaluate dual-species biofilm formation with the rest of the *E. coli* O157:H7 strains in Table 5.2.

Table 5.1 List of environmental bacteria isolates.

	Strain	Species	Bilfilm formation [a]
Gammaproteobacteria	FC2203	*Acinetobacter genospecies 3*	+
	FC1169	*Enterobacter cloacae*	+
	FC2134	*Flavimonas oryzihabitans*	++
	FC2202	*Klebsiella pneumoniae*	+++
	FC1161	*Pantoea agglomerans*	+
	FC3223	*Pectobacterium cypripedii*	-
	FC1136	*Pseudomonas fluorescens*	+
	FC2208	*Rahnella aquatilis*	+
	FC2204	*Stenotrophomonas rhizophila*	+++
Betaproteobacteria	FC1167	*Burkholderia caryophylli*	+++
	FC1138	*Ralstonia insidiosa*	+++
Alphaproteobacteria	FC1140	*Rhizobium radiobacter*	+
Flavobateria	FC1224	*Flavobacterium resinovorum*	+

[a] Biofilm formation capacity of the environmental strains determined using criteria of Stepanović and colleagues (Stepanović *et al*., 2000). +++, ++, +, and - represent strong, moderate, weak, and no biofilm formers, respectively.

Table 5.2 List of *E. coli* O157:H7 strains.

Strain	Alias	Source of Isolation	Curli Expression [a]	Biofilm Formation [b]	Reference or Source
FS4031	C7927	Apple cider	-	-	(Uljas and Ingham, 1999)
FS4052	ATCC 43888 /pGFP	Re: FS4108	+	-	(Fratamico et al., 1997)
FS4057	RM 1918	Outbreak (lettuce)	-	-	R. Mandrell
FS4060	RM 4406	Outbreak (lettuce)	+	++	R. Mandrell
FS4108	ATCC 43888	Human feces	+	-	ATCC c
FS4155	EC869	Cattle kidney	-	+	M. Mammel
FS4157	EC4115	Human, spinach outbreak, 2006	-	-	M. Mammel

[a] The curli expression was defined as positive (+) or negative (-) according to the colony color on Congo Red Agar.

[b] Biofilm formation capacity of *E. coli* O157:H7 strain. +++, ++, +, and - represent strong, moderate, weak, and no biofilm formers, respectively (Stepanović *et al.*, 2000).

[c] American Type Culture Collection.

5.2.2 Biofilm formation

Single or dual-species biofilm formation was carried out using three platforms depending on the purposes of the experiment: polystyrene 96-well microtitre plates (BD Biosciences, San Jose, CA) for evaluating biofilm formation potential of each *E. coli* O157:H7 or environmental strain by measuring biomass attaching to the microplate surface; 12-well polystyrene tissue culture plates (BD Biosciences) for enumerating bacterial cell counts in biofilms; and 50 mm Petri dishes with 25 mm glass-bottom (MatTek Co., Ashland, MA) for examining biofilms by fluorescence microscope. Cultures of individual tested strains were aerobically grown overnight in tryptic soy broth (TSB), washed three times in phosphate buffered saline (PBS, pH7.2), and then adjusted in 10% TSB to approximately 10^9 CFU/ml as original inocula. When using 96 well microtitier plates, 200 µl media was added in each well, and for 12 well tissue plates and petri-dish plates 3 ml media was used. An inoculum was diluted 1:100 in 10% TSB in appropriate vessels and incubated at 30 ^oC for 24 or 48 hrs with moderate agitation (80 RPM/min orbital shaking) to allow biofilm formation. For dual-species biofilms, the inoculation volume of each strain was equal to the inocula (10^9 CFU/ml) for monoculture biofilm inoculation.

5.2.3 Biomass quantification

Total biomass of mono- and dual-species biofilms was determined using crystal violet staining assay (Stepanović *et al.*, 2000) with modification as previously described (Liu *et al.*, 2013).

5.2.4 Bacterial cell enumeration

For the enumeration of bacteria cells in biofilms formed on 12-well tissue culture plates, the liquid culture was removed and the wells were rinsed with 3 ml sterile PBS for 3 times. Cells remaining attached to the culture plate surface were harvested by uni-directional scraping with a sterile cotton-headed stick and released into 10 ml sterile PBS by rigorous twirling and squeezing against vial side. The resulted samples were then vortexed vigorously for 1 min to facilitate cell dispersion. Cell suspension was 10-fold serially diluted and plated on Tryptic Soy Agar (TSA) and sorbitol MacConkey agar (SMAC) for enumerating cells of the environmental isolates and *E. coli* O157:H7 strains, respectively.

5.2.5 Fluorescence microscopy

GFP expressing *E. coli* O157:H7 (FS4052) and *R. insidiosa* (FC1138) strains, individually or in combinations, were incubated in glass-bottomed Petri dish as described above for 48 hrs to allow the formation of single or dual-species biofilms. After removal of culture suspension, biofilms on the glass bottom were rinsed three times using sterile PBS and stained for 20 min using red fluorescent dye SYTO 61 (Life Technology Co., Grand Island, NY.). Excessive dye was then removed, followed by rinsing 5 times with PBS. The stained biofilms were examined under a Zeiss AxioObserver100 fluorescence microscope (Oberkochen, Germany) using Z-stack scanning following deconvolution to subtract background noise. *R. insidiosa* was stained red, while the *E. coli* O157:H7 strain (FS4052) remained green due to GFP expression.

5.2.6 Statistics

Three replications were conducted for each experiment and statistic analyses were performed using Student's t-test or one-way ANOVA (Prism 5; GraphPad, La Jolla, CA). Data analysis methods are described in tables and figures. Treatments were considered significantly different when a P-value was less than 0.05.

5.3 Results

5.3.1 Dual-species biofilms formed by environmental isolates and *E. coli* O157:H7

The total biomass of dual-species biofilms formed by each of the environmental strains in the presence of *E. coli* O157:H7 strain FS4052 was compared to that of the respective monoculture biofilms (Figure 5.1). *Flavimonas oryzihabitans* strain FC2134, *Klebsiella pneumoniae* strain FC2202, *Stenotrophomonas rhizophila* strain FC2204, *Burkholderia caryophylli* strain FC1167, and *Ralstonia insidiosa* strain FC1138 were categorized as moderate or strong biofilm formers, based on the measurements of the biomass production in monoculture biofilms. When co-cultured with *E. coli* O157:H7, three of these strains, FC2134, FC1167, and FC1138, exhibited significantly higher ($p<0.05$) biomass production in dual-species biofilms than that in the monoculture biofilms of either environmental strain or *E. coli* O157:H7 strain FS4052. The biomass increase ranged from 63 to 180%. Three of the environmental isolates with weak biofilm formation potentials, *Acinetobacter* spp. strain FC2203, *Pectobacterium* spp. strain FC3223, and *Rhizobium radiobacter* strain FC1140, showed a slight increase of total biomass in the dual-species biofilms. However, the increase did not alter the overall categorization of these strains as weak biofilm producers. For the other environmental

strains, including both strong and weak biofilm formers, the presence of *E. coli* O157:H7 did not significantly affect the biomass measurements in dual-species biofilms in comparison to respective monoculture biofilms formed by environmental strains.

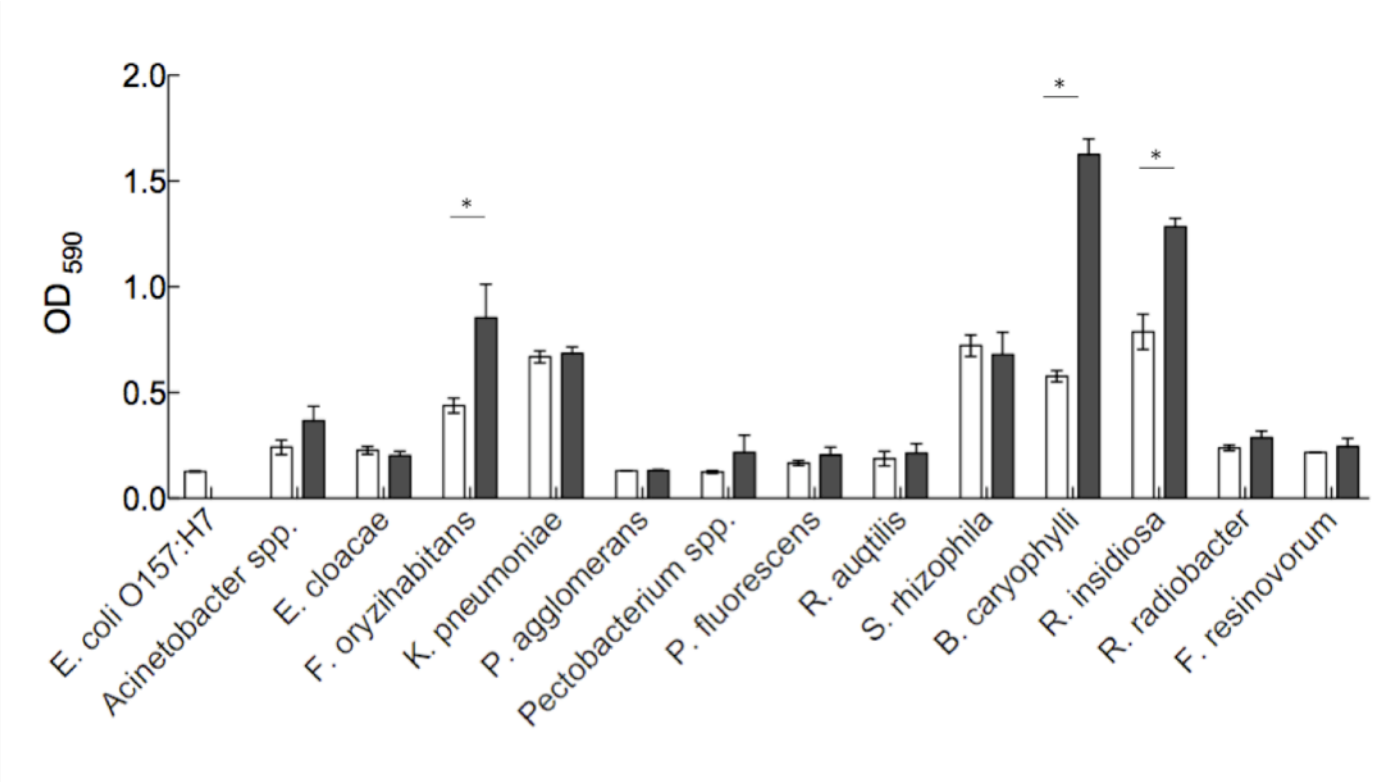


Figure 5.1 Total biomass of mono- and dual-species biofilms formed by environmental bacterial isolates, indicated on X-axis, and *E. coli* O157:H7 (FS4052). Equal volume of inocula for each strain was used in forming monoculture and dual-species biofilm formation. Biomass was determined by absorbance at 590nm after crystal violet staining. ☐ Monoculture biofilms; ■ Dual-species biofilms. Student's t-Test was used to compare the total biomass production of monoculture biofilm formed by environmental bacterial isolate and of dual-species biofilms formed by environmental bacterial isolate and *E. coli* O157:H7. *: Significant difference in biomass between monoculture and dual-species biofilms ($p<0.05$).

Cell numbers of both environmental and *E. coli* O157:H7 strains in single or dual-species biofilms were enumerated by plating (Table 5.3). All of the dual-species biofilms showing enhanced biomass production had an increased incorporation of one of the strains in the combination. For example, *E. coli* O157:H7 cell counts increased significantly ($p<0.05$) by 0.96 log10 unit in dual-species biofilms formed with *R. insidosa* (respectively) in comparison to *E. coli* O157:H7 monoculture. In another instance, *E. coli* O157:H7 cell counts in dual-species biofilms with *B. caryophylli* were 0.93 log10 units higher than that in the monoculture biofilms. In these cases, the presence of *E. coli* O157:H7 in the dual-species biofilms was comparable to that of the strong biofilm forming environmental strains. Cell counts of environmental strains, including, *F. orvzihabitans* ($p<0.05$), *P. cypripedii* ($p<0.05$), *P. fluorescens* ($p<0.05$) and *R. radiobacter* ($p<0.05$), were significantly higher in the dual-species biofilms than in respective monoculture biofilms. In the other combinations, neither environmental strains nor *E. coli* O157:H7 showed significantly increased cell counts in dual-species biofilms compared to the respective monoculture biofilms. A significantly reduced presence of *E. coli* O157:H7 in biofilms was observed when co-cultured with *E. cloacae* ($p<0.05$), *S. rhizophila* ($p<0.05$) or *R. radiobacter* ($p<0.05$), while cell counts for the environmental strains *K. pneumoniae* and *R. aquatilis* in biofilms decreased in the presence of *E. coli* O157:H7. Overall, only 2 strains, *B. caryophylli* and *R. insidiosa*, which were both prolific biofilm producers, strongly promoted the incorporation of *E. coli* O157:H7 into biofilms. Other tested environmental isolates, including strong biofilm former *K. pneumonia*e and *S. rhizophila*, did not significantly increase the presence of *E. coli* O157:H7 in biofilms.

Table 5.3 Cell enumeration for dual-species biofilms (and monoculture biofilm) formed by *E. coli* O157:H7 (FS4052) and different environmental strains.

Environmental Strain	**Cell counts (log CFU/cm^2) in biofilms Dual-species /Monoculture** [a]		**Percentage of *E. coli* O157:H7 in dual-species biofilms (%)** [b]
	***E. coli* O157:H7** [c]	**Environmental strain**	
A. genospecies 3	5.91±0.15 /6.21±0.60	6.68±0.22 /6.52±0.01	14.99±5.37
E. cloacae	5.17±0.12 /5.72±0.20 *	6.24±0.17 /6.35±0.14	8.68±4.29
F. orvzihabitans	5.85±0.11 /5.53±0.43	6.87±0.11 /5.98±0.07 *	9.30±3.72
K. pneumoniae	5.25±0.62 /5.72±0.57	6.87±0.04 /7.01±0.05 *	4.68±6.41
P. agglomerans	5.84±0.57 /6.52±0.20	5.96±0.28 /6.23±0.47	44.09±21.50
P. cypripedii	6.43±0.10 /6.46±0.34	4.52±0.24 /3.25±0.07 *	98.56±1.06
P. fluorescens	5.80±0.08 /5.72±0.20	6.60±0.16 /6.16±0.11 *	13.70±2.84
R. aquatilis	6.18±0.42 /6.58±0.15	6.15±0.04 /6.50±0.21 *	51.16±23.11
S. rhizophila	5.31±0.28 /6.32±0.04 *	7.32±0.24 /7.55±0.30	1.03±0.35
B. caryophylli	6.20±0.46 /5.27±0.26 *	6.51±0.23 /6.91±0.66	33.71±12.32
R. insidiosa	6.80±0.31 /5.84±0.52 *	6.97±0.20 /7.43±0.31	41.56±17.43
R. radiobacter	3.81±0.38 /5.72±0.20 *	6.13±0.18 /5.18±0.32 *	0.51±0.25
F. resinovorum	4.86±0.60 /5.72±0.57	6.61±0.06 /6.57±0.20	3.18±3.64

[a] Equal volume of inocula for each strain was used in forming monoculture and dual-species biofilm formation. Number in front of "/" indicates the cell counts in dual-species biofilms and that following "/" indicates cell counts in respective monoculture biofilms. Student's t-Test was used to compare the cell counts of each strain in their mono- and dual-species biofilms.

[b] Proportion of *E. coli* O157:H7 cells in the total cell counts of the dual-species biofilms.

Percentage (%) = *E. coli* O157:H7 counts (1st column before slash) / [*E. coli* O157:H7 counts (1st column before slash) + environmental bacteria counts (2nd column before slash)]

[c] For the analysis of each environmental strain in forming dual-species biofilms, cells in monoculture biofilms of *E. coli* O157:H7 strain FS4052 was independently enumerated.

* Significant difference ($p<0.05$) in cell counts between dual-species and respective monoculture biofilms.

5.3.2 Dual-species biofilms of *R. insidiosa* with the 6 *E. coli* O157:H7 strains

R. insidiosa was one of the most frequently isolated strong biofilm forming bacterial species from fresh produce processing plants. The finding that *R. insidisa* strain promoted biofilm formation by *E. coli* O157:H7 strain FS4052 suggests a role for this bacterium in the survival and dissemination of foodborne pathogens in food processing environments. To examine if the observed increase of incorporation by *E. coli* O157:H7 in dual-species biofilms formed with *R. insidiosa* was strain specific, *E. coli* O157:H7 strains from diverse sources were tested for forming dual-species biofilms with *R. insidiosa*. Selected *E. coli* O157:H7 strains were first tested for curli production and biofilm formation (Table 5.2). Three of the tested *E. coli* O157:H7 strains expressed curli on CRI plates, including FS4052 and the parental strain ATCC43888 (FS4108), and one outbreak related isolate (FS4060). All but one (FS4060) tested strains were categorized as non- or weak-biofilm formers. Strain FS4060 exhibited moderate biofilm formation in monoculture. Both strain FS4052 and the parental strain FS4108 exhibited similar levels of curli expression and biofilm formation, indicating that the presence of GFP-expressing plasmid had no affect on the biofilm formation of these strains.

Total biomass in the dual-species biofilms with *R. insidiosa* increased for all tested *E. coli* O157:H7 strains in comparison to that of *R. insidiosa* monoculture biofilms, and the increases were statistically significant for 4 of the 6 strains ($p<0.05$, Figure 5.2A) in addition to the strain FS4052. For 5 of the 6 *E. coli* O157:H7 strains, the dual-species biofilms showed significant increases ($p<0.05$) in *E. coli* O157:H7 cell counts compared to respective *E. coli* O157:H7 monoculture biofilms. The increases ranged from 0.7 to1.4 log10 units (Figure 5.2B). These results demonstrated a consistent increased

incorporation of *E. coli* O157:H7 in dual-species biofilms, suggesting a species-specific rather than strain specific interaction between *R. insidiosa* and *E. coli* O157:H7 strains.

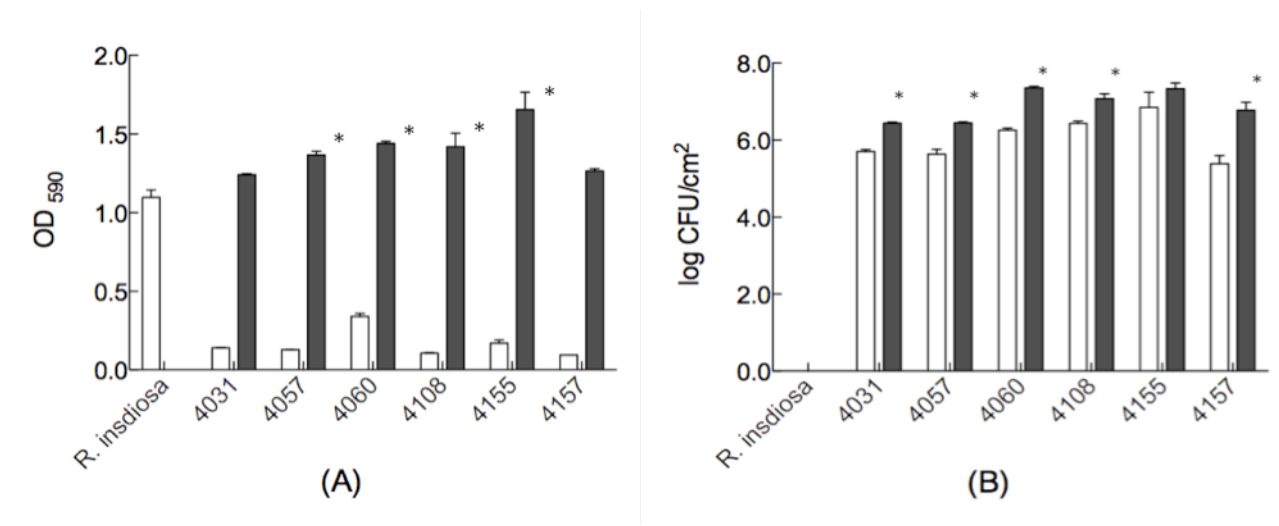


Figure 5.2 Mono- and dual-species biofilms formed by selected *E. coli* O157:H7 strains and *R. insidiosa*. (A) Total biomass in biofilms determined by crystal violet staining assay. One-way ANOVA was used to test the total biomass production of biofilms formed by *R. insidiosa* with or without different *E. coli* O157:H7 strains. Dunnett's multiple comparisons were applied between *R. insidiosa* biofilm and each dual-species biofilms. (B) Cell counts of *E. coli* O157:H7 in mono and dual-species biofilms. Student's t-Test was used compare the cell counts of *E. coli* O157:H7 strain in monoculture and dual-species biofilms. ☐ Monoculture biofilm; ■ Dual-species biofilms.*: Significant difference ($p<0.05$) in total biomass between *R. insidiosa* monoculture biofilms and dual-species biofilms with indicated *E. coli* O157:H7 strain (For A); and in *E. coli* O157:H7 cell counts in dual-species biofilms with *R. insidiosa* and respective *E. coli* O157:H7 monoculture biofilms.

5.3.3 Microscopic examination of dual-species biofilms of *R. insidiosa* and *E. coli* O157:H7

Monoculture and dual-species biofilms formed by *E. coli* O157:H7 strain FS4052 and *R. insidiosa* were examined using a fluorescence microscope (Figure 5.3). The fluorescent images represented cross section views from different angles of singular cut plans cross the biofilms. After 2 days of growth, *E. coli* O157:H7 alone did not form discernible biofilms under conditions used in this experiment. Only scattered attachment of *E. coli* O157:H7 cells were found on the glass surface (Figure 5.3A). In contrast, *R. insidiosa* alone attached on the glass and formed a biofilm with extensive clustering of cell masses resembling microcolonies (Figure 5.3B). In the dual-species biofilms, *E. coli* O157:H7 cells were seen more evenly attached on glass surface or on top of *R. insidiosa* (Figure 5.3C). There appeared a tendency of *E. coli* O157:H7 cells to co-localize with those of *R. insidiosa*. The increased presence of *E. coli* O157:H7 cells in the dual-species biofilms was accompanied by an apparent thickening of the biofilms. The thickness of the biofilm increased from around 10 μm for the monoculture of *R. insidiosa* to more than 20 μm for the dual-species biofilms. The observed thickening of the dual-species biofilms was consistent with the increased biofilm biomass, when *E. coli* O157:H7 strains were co-cultured with strong biofilm formers such as *R. insidiosa* and *B. caryophylli.*

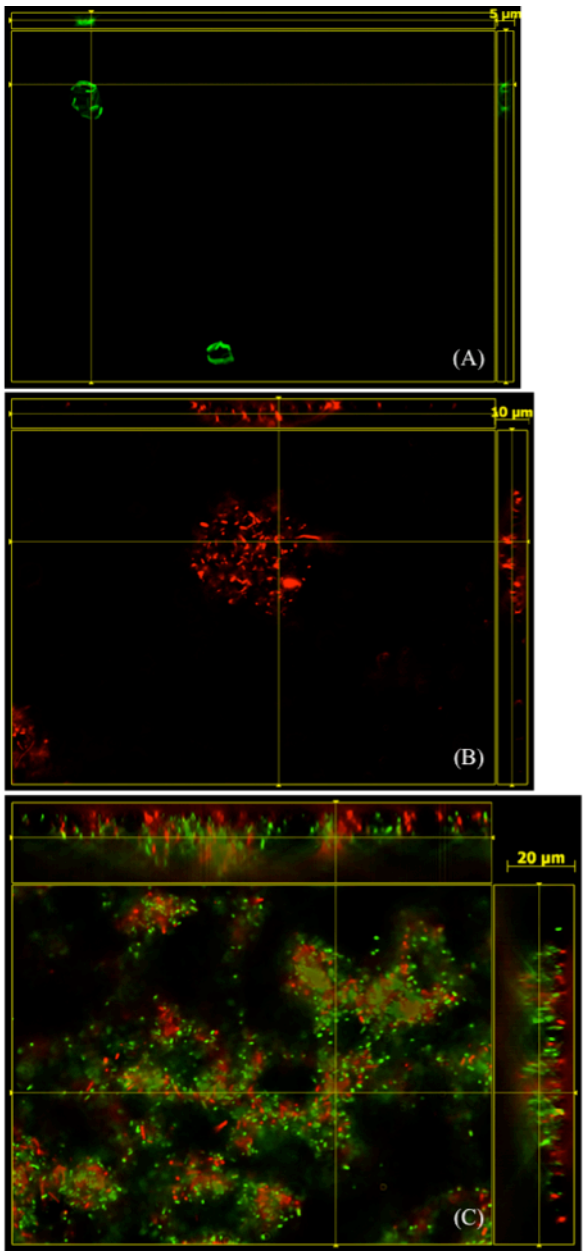


Figure 5.3 Fluorescence microscopy of monoculture and dual-species biofilms formed by *E. coli* O157:H7 (FS4052) and *R. insidiosa*. The main panel showed a cross section view from the top and side panels showed cross section views from the sides of the biofilms within the Z-stack. The cut lines were shown. Lines inside the panels indicate the coordinates of the cross sections. (A) Monoculture biofilm of *E. coli* O157:H7; (B) Monoculture biofilm of *R. insidiosa*; (C) Dual-species biofilms.

5.4 Discussion

By using abiotic surface as substratum, we demonstrated that environmental bacterial strains isolated from fresh-cut processing plants, including strong biofilm formers *R. insidiosa* and *B. caryophylli*, promoted the incorporation of *E. coli* O157:H7 into dual-species biofilms. In contrast, some other prolific biofilm forming isolates, such as *S. rhizophila* and *K. pneumoniae,* failed to promote *E. coli* O157:H7 biofilm formation. In addition, enhanced biomass production in dual-species biofilms produce by *R. insidiosa* and *E. coli* O157:H7 suggested an interwoven interaction between these two strains. Although the mechanisms of this effect are not clear, this interaction seems to be species specific. Similarly, Castonguay and colleagues observed that *E. coli* PHL565 cell counts in biofilms were significantly higher in the presence of *Pseudomonas putida* but not *Staphylococcus epidermidis,* suggesting a species specific interaction between *Pseudomonas putida* and *E. coli* PHL565 (Castonguay *et al.*, 2006). However, in a study using bacteria strains isolated from a beef processing plant Marouani-Gadri and colleagues (Marouani-Gadri *et al.*, 2009) reported a general increase of *E. coli* O157:H7 cell counts in biofilms formed with all but one of the isolates. Data obtained in this study support the notion that only certain bacterial species enhance the biofilm formation by *E. coli* O157:H7.

The expression of curli by *E. coli* is correlated to the biofilm forming potential (Ryu *et al.*, 2004). However, neither curli production nor biofilm forming ability of *E. coli* O157:H7 has been shown to correlate to the potential of pathogenesis in causing foodborne outbreaks. None of the 16 *E. coli* O157:H7 strains isolated from fresh produce related outbreaks in 2006 showed strong curli expression or biofilm formation (Uhlich *et*

al., 2008). Similarly, we observed that the majority of *E. coli* O157:H7 strains used in this study, which were isolated from various outbreaks, did not express curli and showed weak or non-biofilm formation. Therefore, curli expression and strong biofilm formation are more likely exceptions among *E. coli* O157:H7 strains. Since all but one of tested *E. coli* O157:H7 strains responded to the presence of *R. insidiosa* with increased incorporation in biofilms, it is reasonable to speculate that the enhanced biofilm forming abilities of *E. coli* O157:H7 strains were due to the production of curli. Therefore, each *E. coli* O157:H7 strain recovered from dual-species biofilms was plated on Congo red agar to test their curli production. However, results showed that co-culturing with *R. insidiosa* could not alter *E. coli* O157:H7 curli production (data not shown). Findings in this study support the plausibility that synergistic interactions with strong biofilm forming members of native microflora, such as *R. insidiosa*, might play an important role for the survival and dissemination of *E. coli* O157:H7 in food processing environments such as various surfaces used in fresh produce processing.

R. insidiosa and a closely related species, *R. pickettii*, are common industrial contaminant that has been isolated from water bodies, soil, water distribution system, and laboratory purified water systems (Ryan *et al.*, 2011). In our recent sampling of fresh produce processing plant, *R. insidiosa* was the most represented species isolated from various food contact and non-food contact surfaces (Liu *et al.*, 2013). We observed that all *R. insidiosa* isolates obtained in the previous study were moderate or strong biofilm producers. The finding that *R. insidiosa* strongly promoted the incorporation of *E. coli* O157:H7 in biofilms suggests that forming heterogeneous biofilms with *R. insidiosa* or related bacterial species would be an effective surviving strategy for pathogenic bacteria

such as *E. coli* O157:H7. Therefore, better understanding of the interactions between enteric pathogens and plant related bacteria could provide new insights for a potential survival route of human pathogens in fresh and fresh-cut produce processing environment.

5.5 Conclusion

While the majority of the gram-negative bacteria isolated from fresh-cut processing plants did not promote the incorporation of *E. coli* O157:H7 in dual-species biofilms, two species, *B. caryophylli* and *R. insidiosa*, both identified as potent biofilm producers, significantly increased the numbers of *E. coli* O157:H7 in the respective dual-species biofilms. Moreover, the thickness of dual-species biofilm formed by *R. insidiosa* and *E. coli* O157:H7 was more than the sum of the respective monoculture biofilms, indicative of possible interwoven structure contributed by both species. Our data demonstrated that such a protective effect was species-specific, suggesting the need to prevent the growth of these resident bacteria in order to reduce the attachment by *E. coli* O157:H7.

5.6 Acknowledgments

We thank Drs. R.E. Mandrell (USDA ARS WRRC, Albany, CA.) and M.K. Mammel (FDA, Laurel, MD.) for providing some of the strains used in this study.

Chapter 6 Effects of environmental parameters on the dual-species biofilms formed by *Escherichia coli* O157:H7 and *Ralstonia insidiosa* isolated from a fresh-cut processing facility

6.1 Introduction

Biofilm formation by various bacterial species in food processing facilities can be a vector for food spoilage and hence a food safety concern (Pérez-Rodríguez *et al.*, 2008), especially given the ability of biofilms to provide protection of pathogenic bacteria against environmental stresses, such as low temperature and disinfectant treatments (Bore and Langsrud, 2005). Additionally, it has been demonstrated that pathogenic strains can grow on biofilms formed by environmental bacteria isolated from meat and dairy processing facilities (Chmielewski and Frank, 2003). In chapter 4, a species specific interaction between *Escherichia coli* O157:H7 and *Ralstonia insidiosa*, an environmental bacterium isolated from a fresh-cut processing facility, was identified. Dual-species biofilms formed by *E. coli* O157:H7 and *R. insidiosa* displayed significant increases in biomass and thickness in comparison to their respective monoculture biofilms, indicating a potential interaction between the two strains in forming biofilms.

Environmental factors, such as low temperature, continuous fluid flow, and limited nutrient availability, are associated with the phenotypic changes of environmental bacteria from planktonic to attached form (Chmielewski and Frank, 2003). There is substantial microbial growth on fresh-cut produce at low temperature (10 ^{o}C), whereas 4 ^{o}C discourages bacterial proliferation, suggesting that non-refrigeration temperatures allow the growth of microorganisms (Zagory, 1999). A lower temperature could result in biofilm related gene expression in generic *E. coli*, indicating that low temperature is an important cue for the development of *E. coli* biofilm (White-Ziegler *et al.*, 2008). Ryu

and colleague found that low temperature (12 ^{o}C) enhanced the exopolysaccharides (EPS) production in *E. coli* O157 biofilms than those grown at 22 ^{o}C (Ryu and Beuchat, 2004). Extensive application of wash water can result in secondary water flow in environments that are favorable for biofilm formation (Chmielewski and Frank, 2003; Else *et al.*, 2003). The exudates of fresh-cut produce also provide limited nutrients for microbial growth in wash water and on contact surfaces (Burnett and Beuchat, 2001; Capozzi *et al.*, 2009). Bacteral medium made of lettuce juice favored EPS production in aerobically cultured *E. coli* O157:H7 biofilm in comparison to either minimal nutrient salt or rich medium TSB (Ryu and Beuchat, 2004). Lastly, the organic molecules and charged ions presented in fresh-cut residuals or debris might pre-condition the equipment surfaces and turn the surface in favor of bacteria adherence (Chmielewski and Frank, 2003; Simoes *et al.*, 2010b).

However, the effects of environmental factors on the presence of pathogenic strains in dual-species biofilms formed by environmental bacteria and human pathogens remain largely unknown. Since *E. coli* O157:H7 requires a colonizing partner to form biofilms (Klayman *et al.*, 2009), there is a need to characterize the effects of environmental conditions on the growth of *E. coli* O157:H7 in dual-species biofilms. In the present study, marginal temperature abuse (10 ^{o}C), three types of nutrient sources, and a dynamic culture system mimicking secondary water flow in a food processing facility were employed to evaluate the impact on the behavior of *R. insidiosa* and *E. coli* O157:H7 in dual-species biofilms.

6.2 Materials and Methods

6.2.1 Bacterial strains

E. coli O157:H7 strain FS 4052, a weak biofilm former (Chapter 4) derived from the non-pathogenic isolate from bovine feces (ATCC 43888, CDC-B6914) (Fratamico *et al.*, 1997), was used for the studies. It harbors a stably maintained plasmid encoding for green fluorescence protein (pGFP). *R. insidiosa* strain FC1138, a strong biofilm producer isolated from a local fresh produce processing facility was the other strain used in tests. Both strains were maintained at -80 ^{o}C until use. Tryptic soy agar and broth (TSA, TSB, BD Biosciences, San Jose, CA) were the general media for strain enumeration and propagation.

6.2.2 Temperature and media

To test the effect of temperature on biofilm formation, bioiflms were formed at two temperatures (30 and 10 ^{o}C) in 1/10 TSB for 24h or 20 days, respectively. To explore the supportive role of *R. insidiosa* on incorporating *E. coli* O157:H7 in dual-species biofilms in nutrient limited media, three different types of media with various compositions, including diluted (10%) TSB, M9 minimal medium (BD Biosciences, San Jose, CA) supplemented with 0.4% of glucose, and diluted cantaloupe juice (1.25%) prepared from fresh cantaloupe were used. The cantaloupe juice was filtered using 0.1 mm nylon filter bags (Nasco, Whirl-Pak®, Fort Atkinson, WI) and pasteurized with 10 kGy E-beam irradiation prior use. Sterilized cantaloupe juice was stored at -20^{o}C prior to use. Very dilute TSB (1% strength) was used for biofilm development in the continuous culture system.

To determine the availability of nitrogen, each media was tested using a nitrogen analyzer (2410 model, PerkinElmer, Waltham, MA). Alternatively, nitrogen was calculated based on the media chemical composition as indicated in manufacturer instructions. Glucose content was also calculated based on the chemical composition as indicated in manufacturer description. To measure total amount of organics in each medium, the chemical oxygen demand (COD) was determined using semi-automated colorimetry method (EPA, 1993). TSA and sorbitol MacConkey agar (SMAC, BD Biosciences, San Jose, CA) plates were used for enumeration of total aerobic counts of the dual-species biofilms and *E. coli* O157:H7, respectively.

6.2.3 Biofilm formation on tissue culture plates

Monoculture and dual-species biofilms were formed on polystyrene 12-well tissue culture plates as described in Chapter 4. For dual-species biofilms, fresh medium was inoculated with overnight cultures of *E. coli* O157:H7 and *R. insidiosa* to a cell density of 10^7 CFU/ml for each strain. The inoculation was incubated at 30^{o}C or 10^{o}C with moderate orbital shaking (80 rpm) for up to 24 hrs or 20 days, respectively, to allow biofilm formation in the microplate wells. Samples were taken at 4, 8, 24 h or 5, 10, 15, 20 days when incubated at 30 or 10 ^{o}C repectively, to assess the progression of biofilm formation. At sampling time, bacterial cell counts for each strain in the biofilm samples were determined as previously described in Chapter 4.

For the quantification of biomass production of the biofilms growing on 12-well tissue culture plates, crystal violet binding assay was used with slight modifications (Stepanović *et al.*, 2000). Aliquots of 3 ml washing buffer, fixing solution, staining solution, or dye solvent was applied in each well throughout the steps to accommodate

the increased volumes in comparison to the method using 96-well microtitre plates. Biomass was estimated using the averages of three replicate absorbance measurements at 590 nm (OD_{590}). The dissolved dye was diluted as needed to keep the OD_{590} reading under 0.8.

6.2.4 Biofilm formation in drip flow biofilm reactor

To test the dual-species bioflims formation between *E. coli* O157:H7 and *R. insidiosa* under condition of secondary water flow, a drip flow biofilm reactor (BioSurface Technologies Corp., Bozeman, MT) was assembled following the manufacturer's instructions. Two types of surfaces, including standard glass slide (Fisherfinest Premium, Thermo Fisher Scientific Inc., Waltham, MA) and glass filter paper (Whatman Grade GF/F, Sigma-Aldrich Co. LLC., St. Louis, MO) supported by glass slides, were used as solid substrates for biofilm formation. Briefly, to start a monoculture biofilm, standard sized solid substrates were placed in the reactor holding chambers and submerged in 15 ml of fresh TSB inoculated with 1 ml of overnight cultures of either *E. coli* O157:H7 or *R. insidiosa*, at around 8.0 log CFU/ml. For dual-species biofilms, 1 ml of each inoculum was transferred in the same chamber. After 4 h incubation at room temperature (22±2 ^{o}C), the inoculated medium was discharged and fresh media (1% TSB) was continuously delivered into each channel at a speed of 2 ml/min using Masterflex® L/S® peristaltic pump (Cole Parmer, Vernon Hills, IL). The solid substrate in the chambers was tilted at a 10^{o} angle to allow fresh medium to flow smoothly across the substrate.

Biofilms on glass coverslips (Thermo Fisher Scientific, Waltham, MA) were subjected to CLSM examination at 4, 40, and 72 hr, or subjected to cell enumeration 72

hr after inoculation, and those on glass fiber filter paper were used for TEM examination. At each sampling point, biofilms on the substratum was rigorously washed with 30 ml sterile PBS before further processing for respective analyses. For cell enumeration, biofilms were sampled by Z-pattern rubbing using a sterile cotton-tipped swab, followed by procedure described above for biofilms formed under static culture conditions.

6.2.5 Spatial distribution examination by using CLSM and TEM

Glass coverslips supported by glass slides were used as biofilm substrata in drip flow continuous culture biofilm reactor. After incubation and staining with red fluorescent dye SYTO 61 (Life Technology Co., Grand Island, NY.) for 45 min followed by washing with 30 ml water, dual-species biofilms formed by *E. coli* O157:H7 and *R. insidiosa* were examined with confocal laser scanning microscopy (CLSM) system using a Zeiss Axio Observer^{TM} inverted microscope with 40x 1.3 NA and 63x 1.4 NA Plan-Apochromat objectives (Carl Zeiss International, Oberkochen, Germany). Two lasers where used simultaneously, a 488-nm argon laser and a 561 diode-pumped solid state laser with a pin hole of 24 μm passing through a MBS 488/561 beam splitter filter with limits set between 495-530nm for detection of *E. coli* O157:H7 (green) and 600-760nm for detection of *R. insidiosa* (red). Z-stack scanning was conducted to reveal the spatial composition of the dual-species biofilms and the 3-D imaging was generated using Zeiss Zen^{TM} 2009 and AxioVision^{TM} software.

Transmission Electron Microscopy (TEM) was used to examine the spatial distribution by observing the vertical sessions of the biofilms. All of the sample preparation was conducted in USDA-ARS Electron and Confocal Microscopy Unit. Brifely, bacteria was fixed for 2 hours at room temperature in 2.5% glutaraldehyde (v/v)

buffered with 50 mM sodium cacodylate and post fixed in 1% osmium tetroxide (v/v) buffered with 50 mM sodium cacodylate buffer for 2 hours at room temperature. Samples were dehydrated through a graded ethanol series and double distilled H_2O (30 minutes each 20%, 40%, 60%, 80%, 90%, and 100% x 3 [v/v]), infiltrated with Spurrs low-viscosity embedding resin (Spurr, 1969) and ethanol (4 hours each 20%, 40%, 60%,80%,and 100% x3 [v/v]), and polymerized at 60C for 48 hours. Ninety nm thick sections were cut on a Reichert A/O Ultracut microtome using a diamond knife (Diatome) and mounted on 400 mesh Ni grids. Sections were stained with 4% aqueous uranyl acetate (w/v) for 15 minutes followed by 3% aqueous lead citrate (w/v) for 5 minutes. Specimens were viewed with a Hitachi HT 7700 TEM (Hitachi High Technologies America) and the images captured with an AMT XR-41C 4 megapixel camera.

6.2.6 Statistics

Each data point had three replicates throughout the experiments. Student's t-Test was performed to compare the bacteria counts obtained in monoculture and dual-species biofilms. Results acquired from the time course study were subject in two-way ANOVA to elucidate the effect of time and culture method on total bacteria counts in biofilms. Details of data analysis are described in tables and figures. Significant differences were assumed when P-values were less than 0.05. All data was analyzed using Prism 5 software (GraphPad, La Jolla, CA).

6.3 Results

6.3.1 Enhanced incorporation of *E. coli* O157:H7 in dual-species biofilms at 30 ^{o}C and 10 ^{o}C

E. coli O157:H7 and *R. insidiosa* cell counts in dual-species biofilms formed in 10% TSB over a period of 24 hours at 30 ^{o}C (Figure 6.1A) and of 20 days at 10 ^{o}C (Figure 6.1B) were compared to those in respective monoculture biofilms. At 30 ^{o}C, the cell count of *E. coli* O157:H7 in dual-species biofilms was significantly greater in dual-species biofilms than in *E. coli* O157:H7 monoculture after 8-hour incubation (Figure 6.1A). A slight further increase was observed after 24 hours 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 biofilm and dual-species biofilms (Figure 6.1A). *E. coli* O157:H7 incorporation into dual-species biofilms followed similar patterns when incubated at 10 ^{o}C (Figure 6.1B). After one day, *E. coli* O157:H7 cell count in dual-species biofilms was comparable to that in monoculture biofilm. The increased cell counts of *E. coli* O157:H7 in dual-species biofilms started from 5 days and lasted till 20 days after inoculation at 10 ^{o}C (Figure 6.1B).

6.3.2 Dual-species biofilm formation in different nutrient limited media

Nitrogen, glucose content and chemical oxygen demand (COD) of fresh media are listed in Table 6.1. The enumeration results showed that *E. coli* O157:H7 strain FS4052 exhibited very weak biofilm forming potential, while *R. insidiosa* strain FC1138 showed strong biofilm formation (data not shown), in all tested media. Moreover, increased cell counts of *E. coli* O157:H7 in dual-species biofilms as compared to monoculture were observed for all tested media (Table 6.1).

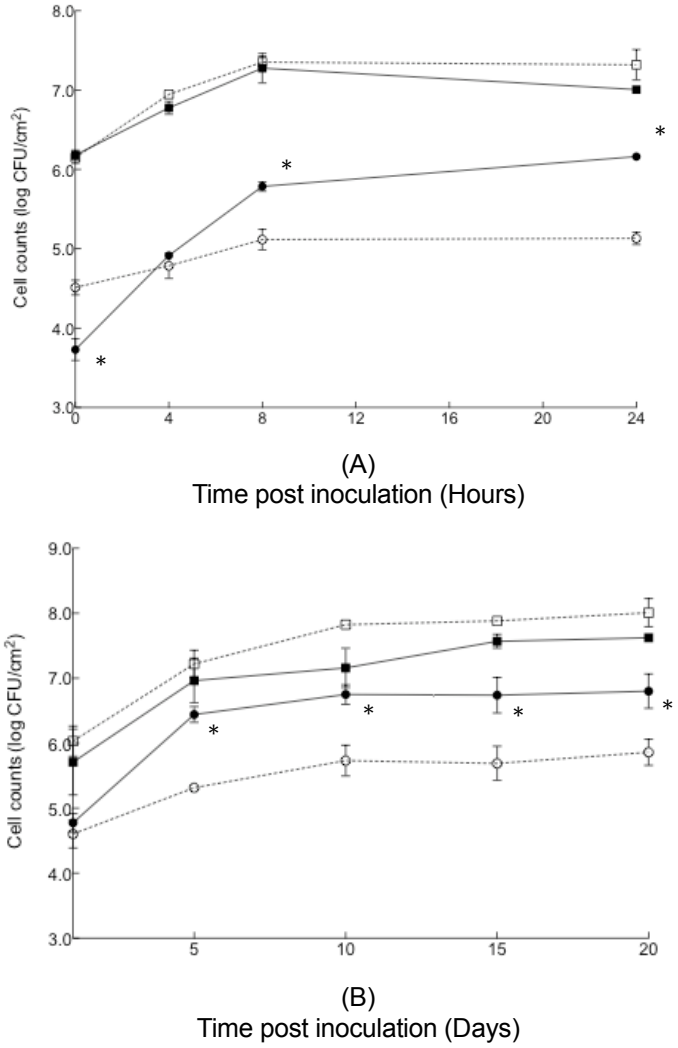


Figure 6.1 Bacterial cell counts of *E. coli* O157:H7 and *R. insidiosa* in dual-species biofilms in static culture. (A) Cell counts of *E. coli* O157:H7 and *R. insidiosa* in biofilms grown at 30 ^{o}C; (B) Cell counts of *E. coli* O157:H7 and *R. insidiosa* in biofilms grown at 10 ^{o}C. Initial inoculation in 10% TSB was 10^7 CFU/ml for both *E. coli* O157:H7 strain FS4052 and *R. insidiosa* strain FC1138. *E. coli* O157:H7 in monoculture biofilm; *E. coli* O157:H7 in dual-species biofilms; *R. insidiosa* in monoculture biofilm; *R. insidiosa* in dual-species biofilms. The two variables in Two-way ANOVA were culture method (mono- or dual-species biofilms) and incubation time. Asterisk represented significant difference between the cells counts of *E. coli* O157:H7 in monoculture and dual-species biofilms ($p<0.05$).

Table 6.1 Characteristics of the media and enumeration of *E. coli* O157:H7 in mono- and dual-species biofilms formed by *R. insidiosa* and *E. coli* O157:H7 4052 after incubating at 30 ^{o}C for 24 hours

Media type	Cell counts of *E. coli* O157:H7 in biofilms (log CFU/cm^2)		Nitrogen content (g/L)	Glucose content (g/L)	COD (mg/L)
	Monoculture	Dual-species			
M9	5.81±0.09	7.11±0.27 *	0.26	4.0	3853±333
10% TSB	5.29±0.50	6.94±0.08 *	0.23-0.28	0.25	2891±231
1.25% CJ	4.91±0.07	7.38±0.15 *	0.09±0.01	NA	1100±174

NA: not available. Asterisk represented significant difference between the cells counts of *E. coli* O157:H7 in monoculture and dual-species biofilms ($p<0.05$).

6.3.3 Dual-species biofilm formation in continuous culture system

The cell counts of *E. coli* O157:H7 and *R. insidiosa* in monoculutre and dual-species biofilms after incubation for 72 h are shown in Figure 6.2. As can be seen, cell counts of *E. coli* O157:H7 in the dual-species biofilms increased by 3.0 log10 units compared to counts for the monoculture biofilm. There was no significant difference in the cell counts of *R. insidiosa* between monoculture and dual-species biofilms.

Time-based profiles of the dual-species biofilm strucutres are shown in Fig 6.3. After 4 hours, aggregates of *R. insidiosa* cells were seen sporadically attached to glass surface and formed microcolony-like structure of less than 5 μm in thickness. A very small number of *E. coli* O157:H7 cells were occasionally observed in these structures (Figure 6.3A). After 40 hours, *R. insidisa* and *E. coli* O157:H7 cells formed discrete mushroom-like domed structures that elevated to approximately 20 μm (Figure 6.3B). *E. coli* O157H7 cells co-localized with *R. insidiosa* cells in these structures. At 72-hour, dual-species biofilms with flat surface were formed. The thickness of the biofilm reached approximately 50 μm. Under the apparent uniform surface there were interconnected domed hallow spaces that were not stained by the STY61 fluorescent dye. *E. coli* O157:H7 cells were predominately found located at the bottom of the biofilms, whereas *R. insidiosa* cells were mainly located at the top of the biofilms (Figure 6.3C). Hence, distinct boundaries were seen between the two strains.

TEM observations generally support CLSM findings (Figure 6.4). Using TEM, *E. coli* O157H7 cells were readily distinguishable from *R. insidiosa* based on cell size and the absence of lipid exclusion bodies (Figure 6.4A and B). *E. coli* O157:H7 and *R. insidiosa* were seen to form mutually exclusive layers in the mushroom-like structures in

the dual-species biofilms (Figure 6.4C); while cells of the two bacteria were generally interspersed at the valleys among the elevated domed structures (Figure 6.4D).

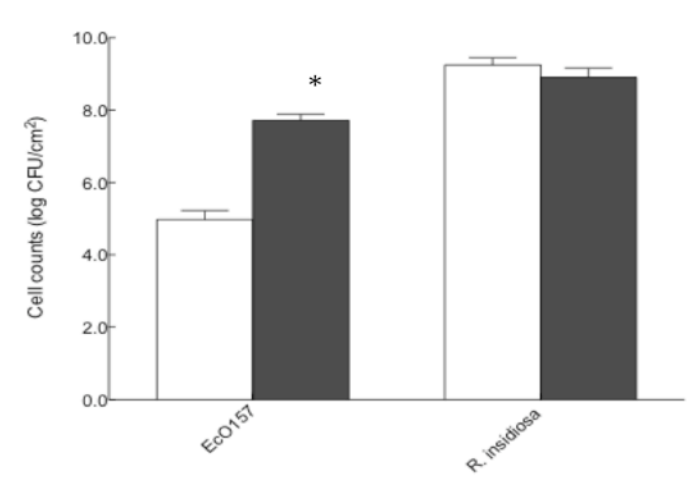
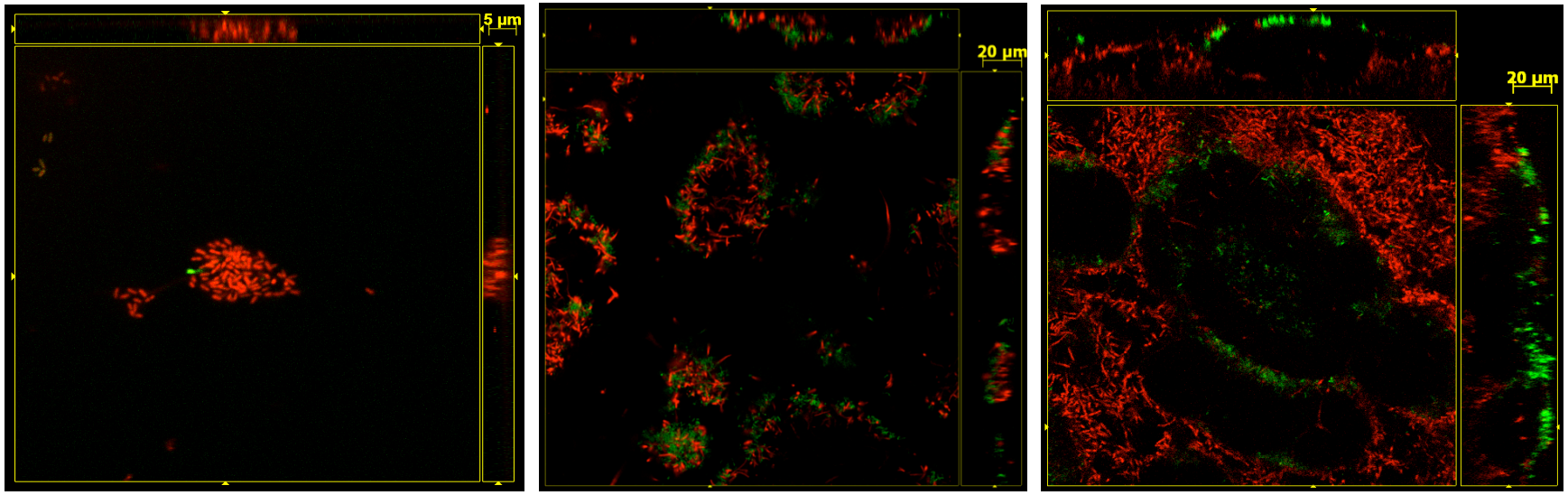


Figure 6.2 Cell counts of *E. coli* O157:H7 and *R. insidiosa* in biofilms developed using drip flow biofilm reactor following 3 days incubation at room temperature. Initial inoculation in TSB was 10^7CFU/ml for *E. coli* O157:H7 strain FS4052 and *R. insidiosa* strain FC1138 followed by continuous culture with 1% fresh TSB. ☐ Monoculture biofilm; ▮ dual-species biofilms. Asterisk represented significant difference between the cells counts of *E. coli* O157:H7 in monoculture and dual-species biofilms ($p<0.05$).



(A) (B) (C)

Figure 6.3 CLSM images showed the development of dual-species biofilms formed by *E. coli* O157:H7 strain FS4052 and *R. insidiosa* after co-inoculation for 4h, 40h and 72 h. Cells were stained with red fluorescent dye SYTO 61. *E. coli* O157:H7 cells remained green due to strong expression of GFP. For each image, main panel showed the top cut-view of the biofilms and the side panels showed cut-views of a cross section of the biofilms.

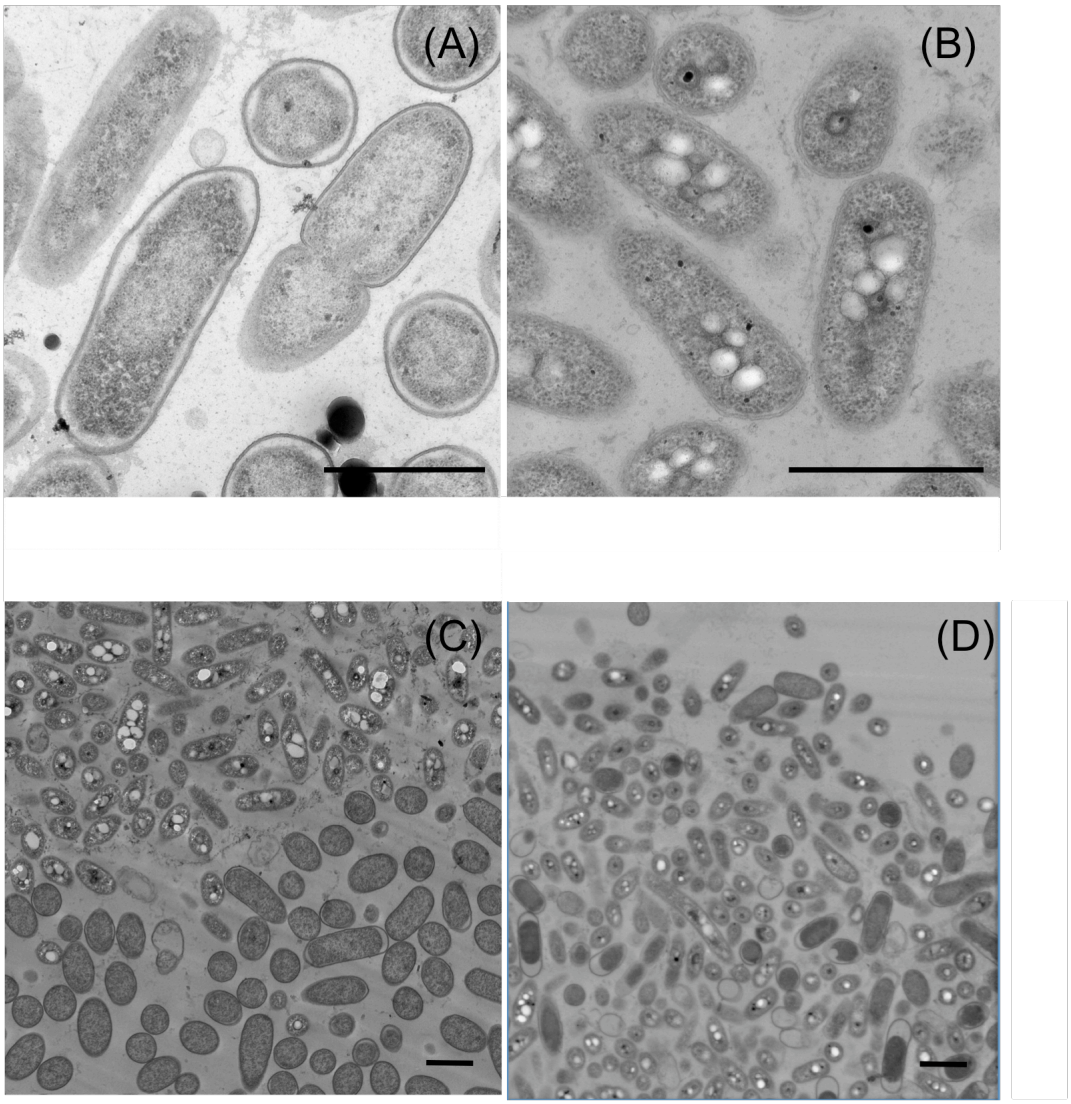


Figure 6.4 TEM images of monoculture and dual-species biofilms formed by *E. coli* O157:H7 (FS4052) and *R. insidiosa* alone or in combination. The solid substrate for biofilm production was located at the bottom of the picture. (A) *E. coli* O157:H7 in monoculture biofilm; (B) *R. insidiosa* in monoculture biofilm; (C) separation of the two strains in the middle of the mushroom-like structure in dual-species biofilms; (D) mixture of the two strains in the valleys between the mushroom-like structures. The scale bar indicated 1 μm.

6.4 Discussion

In the present study, it was found that increased incorporation of *E. coli* O157:H7 in dual-species biofilms could occur at marginal temperature abuse (10 ^{o}C), with diverse culture media and in continuous culture system. The presence of *R. insidiosa*, an isolate from a fresh-cut processing facility, was of great assitance on this enhancement. Of note, a distinct spatial distribution of the dual-species biofilm was observed, where *E. coli* O157:H7 stayed at the bottom in biofilms. It suggested a pertective role of the biofilms structure on the survival of *E. coli* O157:H7. Therefore, biofilm can be a food safety concern in fresh-cut processing facilities.

The US Food and Drug Administration (FDA) requires the temperature in fresh-cut produce processing facilities to be maintained at 4 ^{o}C or lower (FDA, 2008). However, relatively elevated temperatures, which may result from local heat sources or non-uniform temperature distributions, can be conducive to bacterial proliferation in processing facilities. Results acquired from this study show that resident biofilm former, *R. insidiosa*, could increase the number of *E. coli* O157:H7 at marginal temperature abuse conditions (10 ^{o}C), whereas refrigeration temperature completely discouraged bacteria growth and biofilm formation (data not shown). This observation suggested that the interaction between *E. coli* O157:H7 and *R. insidisa* was not temperature dependent; nevertheless, permissible temperature for cellular metabolism was essential for this process. Although most studies concerning human pathogens in biofilms have been conducted at room temperature or higher (Castonguay et al., 2006; Habimana, Heir, et al., 2010; Jones and Bradshaw, 1997; Klayman et al., 2009), results from a study simulating beef slaughtering facility temperature (15 ^{o}C) were consistent with this study. It reported

that all but one environmental isolate could enhance the presence of *E. coli* O157:H7 in dual-species biofilms (Marouani-Gadri *et al.*, 2009).

In fresh-cut processing facilities, equipment surfaces should be clean following cleaning and sanitation operations. However, there might be limited nutrients available for microbial growth in random, hard to clean, areas (Parish, 1997). In addition, the nutrients released from fresh produce during processing periodically provide carbon and nitrogen source for microbial growth. It has been demonstrated that limited nutrient content can promote biofilm formation among a number of bacterial species (Dewanti and Wong, 1995; Jones and Bradshaw, 1997; McAlister *et al.*, 2002; Werner *et al.*, 2004). Three different nutrient sources used in the present study have various nutrient compositions. M9 minimal salt is rich in glucose and nitrogen to support microbial prepergation. Dilute (10%) TSB provided limited glucose (less than 0.99 g/L) (Chapman *et al.*, 1971); but rich nitrogen, and trace amount of vitamins and other inorganic molecules (MacFaddin, 1985). Dilute (1.25%) cantaloupe juice had limited nitrogen content (0.05-0.1 g/L) (Prival and Magasanik, 1971). Additionally, in comparison with the other two media, a lower COD value of dilute cantaloupe juice suggested limited carbon content. Cell counts of *E. coli* O157:H7 were increased in dual-species biofilms in all tested conditions, suggesting that the enhanced incorporation of *E. coli* O157:H7 in dual-species biofilms with *R. insidisa* consistently occurred irrespective of the nutrient profiles of growth media (Table 6.1).

In food processing facilities, secondary water flows over equipment surfaces, such as conveyor belts and interfaces of flumes, commonly generated a low shear force (Kumar and Anand, 1998). The drip flow biofilm reactor is designed to simulate

secondary water flow on surfaces (Goeres et al., 2009); (Simoes, Simoes, Pereira, et al., 2008). Moreover, it also reported that biofilms formed in continuous culture systems could provide spatial distribution of tested bacteria (Bridier, Tischenko, et al., 2011). By using the drip flow biofilm reactor, the spatial arrangement of *R. insidiosa* and *E. coli* O157:H7 in dual-species biofilms was examined (Figure 6.3). Results showed that dual-species biofilm development underwent several steps, including microcolony, mushroom-like structure, and hollow void formation, as the scheme proposed for monoculture biofilm formation (Ma et al., 2009; Stoodley et al., 2002). Various appearances, including microcolonies, mushroom-like structures, and hollow voids, were observed at different stages during dual-species biofilm development. In particular, the spatial arrangement of *E. coli* O157:H7 in biofilms was changed dynamically. *E. coli* O157:H7 started with a lower population in comparison to *R. insidiosa* in microcolonies, but it can grow in the biofilms and its number reached to 8.0 log CFU/cm^2 after 3-day incubation. However, in many cases, *E. coli* O157:H7 was unable to form biofilms in the presence of low shear force, even though biofilms were formed in static culture (Dewanti and Wong, 1995; Klayman *et al.*, 2009).

A number of studies found distinct spatial distributions of *E. coli* O157:H7 in mature dual-species biofilms when *E. coli* was co-cultured with another bacterial species in continuous culture systems. In the present study, *E. coli* O157:H7 co-colonized and formed microcolonies with *R. insidiosa*. Later on, a changed structural relationship was found in mature biofilms. A similar spatial distribution was observed in dual-species biofilms formed by *Acinetobacter* spp. and *Pseudomonas putida* in limited nutrient media with benzyl alcohol as the sole carbon source (Christensen *et al.*, 2002). It was found that

P. putida could utilize benzoate produced by *Acinetobacter* spp. in biofilms where *P. putida* stayed on top of the biofilms whereas *Acinetobacter* spp maintained at the bottom with mushroom-like structures. Therefore, the observed spatial distribution indicated a potential metabolism interaction between *E. coli* O157:H7 and *R. insidiosa*.

Vertical microscopic examination demonstrated different morphologies in mature dual-species biofilms, when different surfaces were used for biofilm formation in drip flow biofilm reactor. A flat biofilm was formed on glass slides, whereas biofilms with mushroom-like structrure were formed on glass fiber surfaces. Kalmokoff and colleague reported that glass fiber filters provided a surface for thicker biofilm formation of *Campylobacter jejuni* (Kalmokoff *et al.*, 2006). This morphologycal variation may be due to different surface properties, such as surface aera and roughness, *et al* (Carlen *et al.*, 2001). Visual evidence gathered from scanning electronic microscopic (SEM) imagines also showed that both monoculture *R. insidiosa* biofilm and dual-species biofilms formed with *E. coli* O157:H7 contained extensive web-like structure (Appendix B). In both tested conditions, *E. coli* O157:H7 might acquire a better protection from surroundings due to the coverage of *R. insidiosa*. These results suggested that surface properties of tested materials had effect on the overall morphology of dual-species biofilms, but had less effect on the cell distribution within the biofilms. It is important to note that a number of parameters, including oxygen availability, bacteria motility, and cell-cell interaction, might impact spatial distribution in dual-species biofilms. More studies are needed to elucidate the specific effects of these parameters that result in different spatial arrangements of *E. coli* O157:H7 in biofilms.

6.5 Conclusion

Enhanced *E. coli* O157:H7 growth was found in dual-species biofilms formed in carbon and nitrogen limited meida (10%TSB) at low temperature (10 ^{o}C) in static culture. With extreme limited nutrient source (1% TSB), dual-species biofilms formed with a distict spatial arrangement of tested bacteria that provide protection on *E. coli* O157:H7 cells. Particularly, surface topography of the solid substrate greatly affected the morphology of dual-species biofilms.

6.6 Acknowledgments

The author thanks Dr. Brendan Niemira for providing E-beam pasteurization treatment for cantaloupe juice used in the present study.

Chapter 7 Potential food safety concern in fresh-cut processing facilities caused by resident biofilm former *Ralstonia insidiosa*

7.1 Introduction

As a recognized vehicle associated with a large number of foodborne outbreaks, fresh-cut produce continues to attract research attention (Harris *et al.*, 2003; Lynch *et al.*, 2009). Various serotypes of *Listeria monocytogenes, Salmonella* spp., and Shiga toxin producing *Escherichia coli* have been identified as sources for fresh produce associated outbreaks (Bowen *et al.*, 2006; Warriner and Namvar, 2010). One potential concern related with the outbreaks is multispecies biofilms formation in processing environments (Carpentier and Chassaing, 2004; Silagyi *et al.*, 2009).

It has been demonstrated in dairy and meat processing facilities that existing native microflora offer a potential transferring pathway for foodborne pathogens (Carpentier and Chassaing, 2004; Lynch et al., 2009; Penteado et al., 2004). Particularly, certain environmental bacteria might play a key role in multispecies biofilms formation by providing protective habitats for planktonic bacteria with enhanced resistance from daily cleaning and disinfections (Jeong and Frank, 1994; Rickard *et al.*, 2003; Van der Veen and Abee, 2011). For instance, *Acinetobacter calcoaceticus*, a strong biofilm former isolated from meat processing facilities, could enhance survival of *E. coli* O157:H7 in a dynamic culture system (Habimana, Heir, et al., 2010). Additionally, *A. calcoaceticus* was found to serve as a "bridge" for other five bacteria isolated from drinking water when forming multispecies biofilms. Absence of *A. calcoaceticus* resulted in 75% reduction of biomass in a 3-day biofilms than that formed by all six species (Simoes, Simoes and Vieira, 2008). Therefore, this type of resident bacteria, a.k.a. bridging bacteria, may not only incorporate pathogenic bacteria species in their biofilms

but also enhanced the biomass production in biofilms, increasing the likelihood of pathogen survival (Uhlich *et al.*, 2010; Van der Veen and Abee, 2011).

As described in Chapter 6, *Ralstonia insidiosa* isolated from fresh-cut processing environment could incorporate *E. coli* O157:H7 into its biofilms under various conditions. This suggests that *R. insidiosa* may be able to incorporate other pathogenic microorganisms into biofilms. The goal of this phase of the research was to evaluate the interaction between *R. insidiosa* and pathogenic species, including *Listeria monocytogenes, Salmonella* spp., and Shiga toxin producing *E. coli*. The key factors that might affect the interactions, such as biofilm-cell attraction, cell-cell adherence and produced metabolite, were tested.

7.2 Materials and Methods

7.2.1 Bacterial strains and media

Bacterial strains used in this study are listed in Table 7.1. *R. insidosa* strain FC1138 was isolated from food contact surface in a fresh-cut processing plant (Liu *et al.*, 2013). *E. coli* O157:H7 strain FS4052 is derivative from the non-virulent strain CDC B6-914 that carries a stable plasmid expressing green fluorescent protein (pGFP) (Fratamico *et al.*, 1997). Other strains of foodborne pathogens were from laboratory stock culture collection. Dilute (10%) tryptic soy broth (TSB, BD Biosciences, San Jose, California) was used for culturing bacteria to form biofilms in static culture and 1% TSB was used for forming biofilms in drip flow biofilm reactor. Tryptic soy agar (TSA, BD Biosciences, San Jose, California), Modified Oxford *Listeria* agar (MOC, BD Biosciences, San Jose, California), Xylose lysine tergitol 4 agar (XLT-4, Neogen, Lansing, MI), and MacConkey agar (sMAC, BD Biosciences, San Jose, California) were

used for enumeration of total bacteria, *L. monocytogenes*, *Salmonella* spp., and Shiga-toxin producing *E. coli*, respectively.

Table 7.1 List of environmental isolate and foodborne human pathogens.

Strain ID	Original Strain Name	Source of Isolation	Reference/Source
FC1138	*Ralstonia insidiosa*	Fresh-cut processing plant	(Liu *et al.*, 2013)
FS2005	*Listeria monocytogenes* 1/2a	Milk	Jeffery Karns
FS2008	*L. monocytogenes*	Milk	Jeffery Karns
FS3022	*Salmonella* newport	Mango	Patricia Millner
FS3060	*S.* poona	Cantaloupe	Jeffery Karns
FS4052	*E. coli* O157:H7	Human feces	(Fratamico *et al.*, 1997)
FS4137	*E. coli* O111:NM	Clinical isolate	Daniel Shelton
FS4140	*E. coli* O45:H2	Clinical isolate	Daniel Shelton
FS4143	*E. coli* O26:H11	Clinical isolate	Daniel Shelton
FS4146	*E. coli* O103:H2	Clinical isolate	Daniel Shelton
FS4178	*E. coli* O104:H4	Clinical isolate	(Al Safadi *et al.*, 2012)

7.2.2 Biofilm formation

Monoculture or dual-species biofilms were produced on 12-well tissue culture plates (BD Biosciences, San Jose, California) followed the procedure described previously (Chapter 5). By using drip flow biofilm reactor (DFB, BioSurface Technologies Corp., Bozeman, MT), monoculture and dual-species biofilms were formed on glass slide and subjected to cell enumeration as described previously (Chapter 6). All biofilms produced on tissue culture plates were incubated at 30 ^{o}C for 24 hr and those grew in drip flow biofilm reactor were kept at room temperature for 3 days.

7.2.3 Pre-conditioned surface with *R. insidiosa* biofilm

In order to test if the enhanced incorporation of *E. coli* O157:H7 in dual-species biofilms (Chapter 6) was mainly caused by the existing *R. insidiosa* biofilm, 1-day *R. insidiosa* biofilms were prepared in 12-well tissue culture plates as described in Chapter 6. After incubation for 24 hrs at 30 ^{o}C, wells with *R. insidiosa* boifilms were washed with PBS for 3 times. Overnight *E. coli* O157:H7 suspension was inoculated on plates with pre-formed *R. insidiosa* biofilms or with *R. insidiosa* on new empty plates. All inoculations were incubated at 30 ^{o}C for another 24h. Biofilms were enumerated at 0, 4, and 24 hrs post inoculation.

7.2.4 Cell-cell adherence

To test whether the effect of *R. insidiosa* biofilm on *E. coli* O157:H7 was dependent upon the physical interaction among the cells, stationary phase culture of *E. coli* O157:H7 and *R. insidiosa* was subjected to modified aggregation study (Simoes, Simoes and Vieira, 2008). Briefly, overnight culture of each strain was harvested by centrifugation (4000×g, 10 min at 4 ^{o}C) and wash three times by Phosphate buffered

saline (PBS, BD, Biosciences, San Jose, California). After the third wash, the pellet was resuspended in PBS and the optical density at 640 nm of the suspension was adjusted to 1.5. Equal amount (1ml) of each strain was gently mixed together at room temperature in 5 ml rolled glass tubes. For the controls made by single strain, 2 ml of each strain was add in 5ml tubes and mixed well. After 24 h at room temperature, the suspension was vortexed for 5s and stained by STYO 61 (Life Technology Co., Grand Island, NY.). Then, the suspension was examined by using Z-stack scanning under fluorescence microscope (Zeiss AxioObserver100, Oberkochen, Germany). *R. insidiosa* was stained red, while *E. coli* O157:H7 remained green.

7.2.5 Cell free pre-conditioned culture media

In order to examine the role of extracellular compounds and metabolites on *E. coli* O157:H7 biofilm formation, cell-free supernatants (grown by *R. insidiosa*, *E. coli* O157:H7 and mixed culture) were used as growth media to cultivate strain FS4052. Briefly, *R. insidiosa* and *E. coli* O157:H7 were inoculated, individually or in combination, to TSB at approximately 10^7cfu/ml and incubated at 30 ^oC for 24 h. After sufficient centrifugation, the supernatant of the culture medium was sterilized by filtering through a 0.2 μm membrane. These cell free pre-conditioned culture media were used separately to support biofilm formation by *E. coli* O157:H7 in static cultures as described above.

7.2.6 Heat inactive treatment of *R. insidiosa* biofilm

To test the effect of extracellular polymeric substances of *R. insidiosa* biofilm on *E. coli* O157:H7 biofilm formation, one-day *R. insidiosa* biofilm was prepared on glass slides in a drip flow biofilm reactor as described in Chapter 6. Biofilm samples with glass

slide were submerged in 80 ^{o}C pre-heated sterile water bath and kept for 30 min to completely inactivate *R. indidiosa* cells (Chmielewski and Frank, 2003; Scher *et al.*, 2005). The glass slide with inactivated *R. insidiosa* biofilms was replaced in the drip flow biofilm reactor and used as substrata for *E. coli* O157:H7 biofilm formation for 72 h as described in Chapter 6.

7.2.7 Biomass quantification and bacteria cells enumeration

Total biomass of biofilms grown on tissue culture plates was quantified by crystal violet binding assay (Chapter 6). To enumerate biofilms formed on the two platforms (tissue culture plate and drip flow biofilm reactor), cell sampling and plating were same as describe previously (Chapter 5 and Chapter 6). Since *R. insidiosa* is a known strong biofilm producer (Chapter 5), biofilm forming capacities of tested pathogens were roughly determined by comparing OD_{590} value with monoculture *R. insidiosa* biofilm.

7.2.8 Statistics

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

7.3 Results

7.3.1 Dual-species biofilms formed by *R. insidiosa* and pathogens

To determine the biofilm forming capacity, total biomass of the monoculture and dual-species biofilms was measured after 24 hr incubation (Table 7.2). The known strong biofilm former, *R. insidiosa*, produced biomass with optical density greater than 2.0 at

590 nm. Biofilms produced by *E. coli* strains FS4140 and FS4143 showed comparable OD values as that of *R. insidiosa* biofilm. Therefore, they were also strong biofilm producers. Two *Salmonella* strains (FS3022, FS3060) and one *E. coli* strain (FS4178) were considered moderate biofilm formers with less than half biomass of *R. insidiosa* biofilm. The rest of tested pathogenic strains (FS2005, FS2008, FS4137, FS4146) were weak biofilm formers as there was lack of biomass accumulation (OD_{590}<0.15).

To elucidate the effect of co-culturing on biomass production in dual-species biofilms, we defined the synergistic effect when the OD value of dual-species biofilms was greater than the sum of the monoculture biofilms of *R. insidiosa* and the pathogenic strain. Significant increases of total biomass in dual-species biofilms were observed when *R. insidiosa* co-cultured respectively with FS2005, FS2008, FS4137, and FS4178. Only a slightly increased biomass production was observed in the respective dual-species biofilms formed by *R. insidiosa* and FS3022 or FS3060, as the increase was not significantly different from the monoculture biofilms.

The total aerobic counts of each pathogenic strain in dual-species biofilms and in monoculture biofilms were determined (Figure 7.1). In monoculture biofilms, the total aerobic counts of pathogenic stains ranged from 5.11 to 7.54 CFU/cm^2. Seven out of 9 pathogenic strains showed significant increases ($p<0.05$) of cell counts in dual-species biofilms comparing with their monoculture biofilms. The increased cell counts were found to range from 0.36 CFU log unit (strain FS4178) to 1.60 CFU log units (strain FS2008). No significant increased cell counts of FS4140 and FS4143, two strong biofilm producers, were observed in the dual-species biofilms.

Table 7.2 Total biomass productions in monoculture and dual-species biofilms formed by foodborne human pathogens and *R. insidiosa*. Student's T-test was used to compare the OD_{590} value of total biomass in dual-species biofilms with the sum of monoculture biofilms in each combination (* $p<0.05$).

Strain ID	**Original Strain Name**	**Monoculture biofilm**		**Dual-species biofilms**
		Pathogen	***R. insidiosa***	
FS2005	*L. monocytogenes* 1/2a	0.019±0.003	2.663±0.208	7.013±0.359 *
FS2008	*L. monocytogenes* 4b	0.028±0.014	2.663±0.208	9.840±3.171 *
FS3022	*S.* newport	0.815±0.124	2.012±0.615	3.292±0.438
FS3060	*S.* poona	0.679±0.042	2.012±0.615	3.225±0.079
FS4137	*E. coli* O111:NM	0.065±0.005	3.121±0.125	3.641±0.214 *
FS4140	*E. coli* O45:H2	3.080±0.338	2.012±0.615	4.064±1.214
FS4143	*E. coli* O26:H11	1.725±0.138	3.121±0.125	4.456±0.361
FS4146	*E. coli* O103:H2	0.147±0.003	3.121±0.125	3.323±0.373
FS4178	*E. coli* O104:H4	0.843±0.109	2.012±0.615	3.803±0.082 *

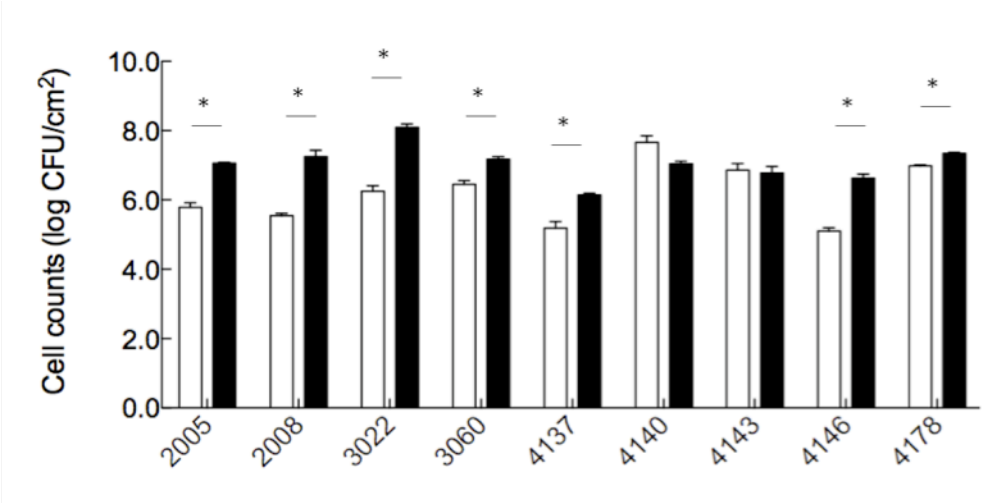


Figure 7.1 Total bacteria counts of tested foodborne human pathogen in monoculture and dual-species biofilms produced with *R. insidiosa*. ☐ Monoculture biofilm grown for 1 day; ■ Dual-species biofilms grown for 1 day. Student's T-test was used to elucidate the statistical relationship between the two columns (* $p<0.05$).

7.3.2 Presence of *E. coli* O157:H7 in biofilms with or without pre-formed *R. insidiosa* biofilm

E. coli O157:H7 cell counts in monoculture, dual-species biofilms with or without pre-formed *R. insidiosa* biofilm were showed in Figure 7.2. At time zero, *E. coli* O157:H7 had a higher number in monoculture biofilm than the dual-species biofilms. After 4 h, the highest cell count of *E. coli* O157:H7 was found in dual-species formed with pre-formed *R. insidiosa* biofilm with a 0.98 log CFU unit increase ($p<0.05$) in comparison to *E. coli* O157:H7 alone. The increased number remained consistent for 24 h, where *E. coli* O157:H7 reached a 1.93 log CFU units of increase ($p<0.05$). Although no significant increase of *E. coli* O157:H7 cell count was observed at 4 h for dual-species biofilms followed by co-inoculating with *R. insidiosa*, a significant increase ($P<0.05$) of *E. coli* O157:H7 (1.01 log CFU/cm^2) was detected after 24 h. This observation suggested that pre-formed *R. insidiosa* biofilm provided a network in dual-species biofilms that could enhance the incorporation of *E. coli* O157:H7 at a faster rate than planktonic *R. insidiosa*.

7.3.3 Cell-cell adherence

It was found that *R. insidiosa* aggregated and formed clusters in the suspension (Appendix C). The *R. insidiosa* clusters in mixed suspensions appeared to sporadically incorporate small amount of *E. coli* O157:H7 cells (Figure 7.3). Moreover, both rod shaped and filamentous *E. coli* O157:H7 cells were found in the clusters.

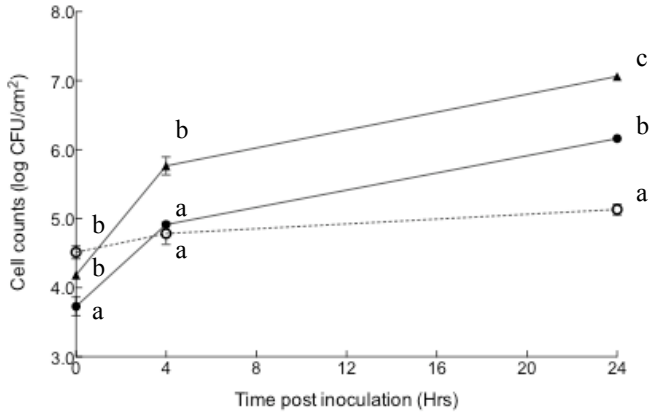


Figure 7.2 Total cell counts of *E. coli* O157:H7 strain FS4052 in monoculture and dual-species biofilms formed with/without the presence of *R. insidiosa*. Monoculture of *E. coli* O157:H7 biofilm; *E. coli* O157:H7 in dual-species biofilms followed by co-inoculation; dual-species biofilm formed by inoculating *E. coli* O157:H7 in 1 day pre-formed *R. insidiosa* biofilm. One-way ANOVA was used to test the effect of different culture on the presence of *E. coli* O157:H7 in biofilms. Tukey's multiple comparison test was used to compare the *E. coli* O157:H7 counts in different biofilms sampled at the same time point ($p<0.05$).

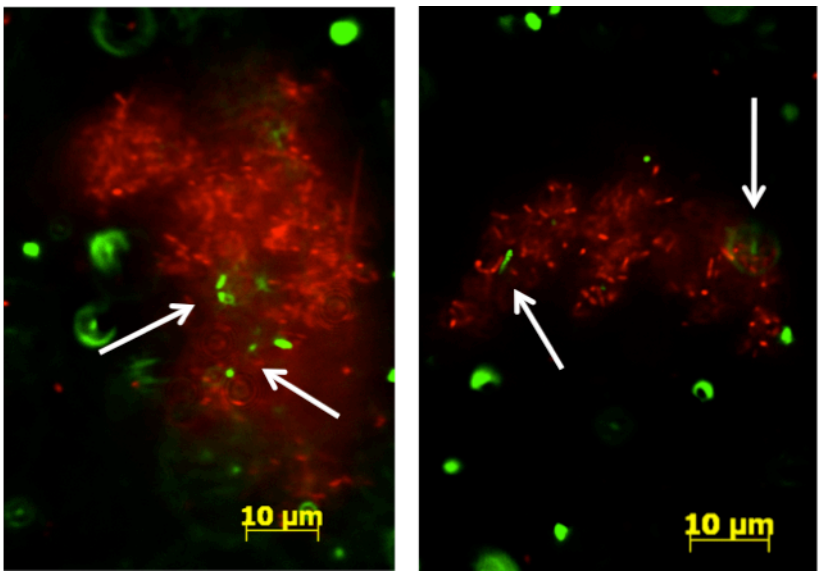


Figure 7.3 Fluorescence microscopy images showed co-aggregation between *R. insidiosa* and *E. coli* O157:H7. Arrows pointed *E. coli* O157:H7 cells that incorporated in the clusters.

7.3.4 Effect of extracellular compounds on *E. coli* O157:H7 biofilm formation

After 24 h of incubation, there was no significant increase of *E. coli* O157:H7 cell counts in the biofilm that grown in pre-conditioned *R. insidiosa* supernatant compared to that in fresh TSB (Figure 7.4). The extracellular compounds and metabolites secreted from *R. insidiosa* did not have a significant effect on *E. coli* O157:H7 biofilm formation. In contrast, when incubating *E. coli* O157:H7 in cell-free *E. coli* O157:H7 supernatant or mixed culture supernatant, the cell counts were significantly reduced.

7.3.5 Effect of heat-inactivated *R. insidiosa* biofilm on *E. coli* O157:H7 biofilm formation

Since previous results suggested that living *R. insidiosa* cells could incorporate *E. coli* O157:H7 in clusters (Figure 7.3), it is important to know if the biofilm matrix with inactivated *R. insidiosa* cells could foster the growth of *E. coli* O157:H7. Plate counting results showed that there was no significant increased growth of *E. coli* O157:H7 on heat inactivated *R. insidiosa* biofilm in comparison to its monoculture biofilm (Figure 7.5). Results acquired from crystal violet staining assay showed a decrease of biomass after heat treatment, although the overall biomass of treated biofilms remained high (Appendix E).

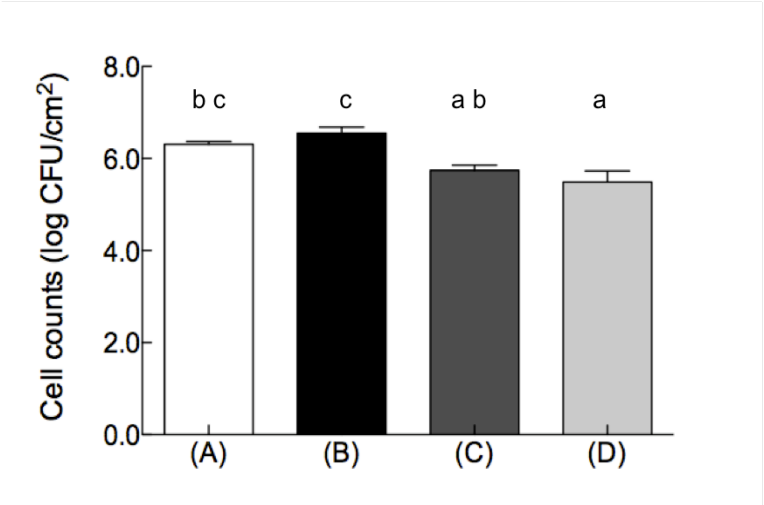


Figure 7.4 Total cell counts of *E. coli* O157:H7 strain FS4052 in monoculture biofilm formed in cell free pre-conditioned TSB after 24 h incubation. (A) in TSB; (B) in *R. insidiosa* supernatant; (C) in *E. coli* O157:H7 supernatant; (D) in mixed culture of *R. insidiosa* and *E. coli* O157:H7. One-way ANOVA was used to test the effect of different pre-conditioned media on *E. coli* O157:H7 biofilm formation. Tukey's multiple comparison test was used to compare the *E. coli* O157:H7 counts in biofilms grown in different culture media ($p<0.05$).

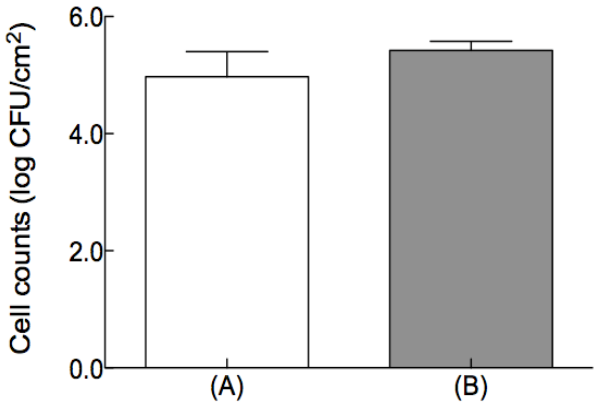


Figure 7.5 Total cell counts of *E. coli* O157:H7 strain FS4052 in monoculture biofilm (A) and biofilm formed on heat-inactivated *R. insidiosa* biofilm (B). Student's T-test was used to compare the *E. coli* O157:H7 cell counts in different biofilm samples ($p<0.05$).

7.4 Discussion

Previously, it was found that the species-specific interaction between *R. insidiosa* and *E. coli* O157:H7 could result in an increased population of *E. coli* O157:H7 in dual-species biofilms (Chapter 5). In this study, results showed that this enhanced incorporation could be extended to other foodborne pathogenic bacteria, including 2 serotypes of *L. monocytogenes*, 2 serovars of *S. enterica*, and 3 serotypes of Shiga toxin-producing *E. coli*. In tested combinations, the increased number of the foodborne pathogen was also accompanied with increased biomass production in dual-species biofilms. Of note, with comparable increased cell counts among different pathogenic bacterial species, drastic elevated biomass accumulations in dual-species biofilms were found in *R. insidiosa-L. monocytogenes* combinations. It suggested that the increased biomass production was not directly related with the increased growth of pathogenic bacteria strains. Although several environmental strains were found to be able to enhance the growth of *L. monocytogenes* in dual-species biofilms (Carpentier and Chassaing, 2004), there are limited reports on the factors that might cause the increased biomass production. Zammer and colleagues found increased biomass production when co-culture *L. monocytogenes* with *Staphylococcus epidermidis*. This increase was related with inocula ratio of the two strains (Zameer *et al.*, 2010). In addition, it has been demonstrated that *L. monocytogenes* biofilm structure could be altered from homogenous to heterogenous with the presence of one *Staphylococcus aureus* strain (CIP 53.156) when examined by SEM (Rieu *et al.*, 2008). Since *R. insidiosa* formed highly heterogenous biofilms alone (Chapter 5, Appendix B), it is reasonable to postulate that a

special structure of the dual-species biofilms formed by *R. insidiosa* and *L. monocytogenes* might exist.

Results acquired in the present study also suggested that *R. insidiosa* might play as a bridging bacterium that is critical for multispecies biofilm formation in fresh-cut processing environments. Increased biomass production was found not only in most of the *R. insidiosa*-pathogen combinations, but also in the dual-species biofilms formed by *R. isndiosa* with environmental strains, isolated from fresh-cut processing facilities (Chapter 4), including *Pantoea agglomerans* and *Rahnella aquatilis* (Appendix D). The ability of *R. insidiosa* to interact with different bacterial species fits the typical characteristics of bridging bacteria (Rickard *et al.*, 2003). For example, *Fusobacterium nucleatum*, a prevalent bacterium in human plaque, could positively interact with bacteria from 7 different genera and thereby connecting the planktonic bacteria to the biofilms and enhanced biofilm production by mediating their surface adhesions (Shaniztki *et al.*, 1998).

Data acquired in the present study suggested that intimate contact with *R. insidiosa* cells is required to increase the incorporation of *E. coli* O157:H7 in dual-species biofilms. As a bridging bacterium, there are two major routes that *R. insidiosa* may incorporate other species. *R. insidiosa* could serve as a primary colonizer attaching on solid substrate and excreting extracellular polysaccharide that provides microniches for secondary colonizers (Rickard *et al.*, 2003). Alternatively, *R. insidiosa* can co-aggregate with other bacteria before the clusters participate on surfaces. Results obtained in the present study supported both functions of *R. insidiosa* on biofilm formation with *E. coli* O157:H7 (Fig 7.2 and Fig 7.3). The inability of *R. insidiosa* supernatant to help *E. coli*

O157:H7 biofilm formation further supported the theory that intimate contact appeared to be critical. This conclusion was consistent with the findings reported by Uhlich and coworkers that direct cell-cell contact with companion strains was required for *E. coli* O157:H7 biofilm formation (Uhlich *et al.*, 2010). Therefore, it is suggested that intimate contact was one of the reasons that caused increased incorporation of *E. coli* O157:H7 in the dual-species biofilms.

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 *R. insidiosa* is capable of communicating with other tested bacteria through signal molecules that subsequently promote multispecies biofilm formation. First of all, *Ralstonia solanacearum*, a phytopathogen, is well known for the production of acyl-homoserine lactones (AHL), the putative cell-cell signaling molecules in biofilm development (Flavier *et al.*, 1997). Secondly, it has been documented that both *E. coli* and Salmonella had the acceptor for AHL molecules in order to detect surrounding microbial community (Dyszel et al., 2010; Michael et al., 2001), theoretically capable of enhancing biofilm formation of these strains. However, a well-designed experiment set up is needed to study the actual interaction.

Combining the fact that *R. insidiosa* was overly represented among bacterial isolates collected from fresh-cut processing facilities, the results acquired in this study raised the prospect that bacterial pathogens capable of forming weak biofilm could interact with *R. insidiosa* to form dual-species biofilms. To our knowledge, this is the first evidence identifying such an environmental strain, *R. insidiosa*, could serve as an aid for the growth of multiple foodborne pathogens in biofilms under mimic produce

processing environments. Moreover, the comparable cells counts of *E. coli* O157:H7 grown on the heat inactivated biofilm and its monoculture biofilms (Figure 7.5) suggested that hot water treatment is sufficient to inactivate the promoted growth of pathogenic strains on existing biofilms. Thus, it can be considered as a recommend operation for cleaning and sanitation practices for fresh-cut processing.

7.5 Conclusion

R. insidiosa, a strong biofilm producer isolated from a fresh-cut processing plant, was found to enhance the growth of various foodborne pathogens, including *L. monocytogenes, Salmonella* spp., and Shiga toxin producing *E. coli*. Intimate cell contacts between *R. insidiosa* and *E. coli* O157:H7 appeared to be an essential criterion. Results suggested that *R. insidiosa* in fresh-cut processing facilities might serve as a bridging bacterium that promotes formation of multispecies biofilms, consequently harboring and assisting the growth of pathogenic microorganisms. Existence of residential strains such as *R. insidiosa* should be regarded as a potential food safety concern in fresh-cut processing facilities in order to minimize cross-contamination.

7.6 Acknowledgements

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Chapter 8 Key Findings and Future Research Recommendations

8.1 Key findings

The resident bacteria recovered from two fresh-cut produce facilities showed biofilm producing capabilities. Among the identified bacteria isolates, *Pseudomonas fluorescens*, *Rahnella aquatilis* and *Ralstonia insidiosa* were the most frequently species isolated from the two sampled processing facilities. Species specific interactions between Gram-negative resident bacteria, including *Burkholderia caryophylli* and *R. insidiosa*, promoted the incorporation of *E. coli* O157:H7 in dual-species biofilms. Such increased incorporation of *E. coli* O157:H7 was reproducible under low temperature (10 ^{o}C) and nutrient limited conditions. A stratified distribution of the two strains, where *E. coli* O157:H7 stayed at the bottome of the biofilms with *R. insidiosa*, was observed when biofilms formed in drip flow biofilm reactor. In additional, the presence of *R. insidiosa* could significantly enhance the incorporation ($p<0.05$) of other human pathogens, including *Listeria monocytogenes*, *Salmonella* spp., and Shiga Toxin producing *E. coli*, when forming dual-species biofilms. Thus, *R. insidiosa* could serve as connecting bacterium embedding foodborne pathogens in biofilms. The presence of *R. insidiosa* might represent potential challenges for reducing food safety risks in fresh-cut processing facilities.

8.2 Future research recommendations

For future research, more studies need to be done to elucidate the key factors resulting the stratified distribution in dual-species biofilms observed by CLSM and TEM. In a review article, Stewaut and Franklin pointed that chemical gradients presented in biofilms is one of the mechanisms related with physiological heterogeneity of bacterial

cells, and intermingled spatial arrangement of bacteria species in such biofilms (Okabe *et al.*, 1999; Stewart and Franklin, 2008). Hence, chemical heterogeneity might play an important role on the spatial arrangement when *R. insidiosa* and *E. coli* O57:H7 form dual-species biofilms in continuous culture system.

Oxygen availability is proposed as a primary factor. Zhang and colleagues demonstrated that the oxygen content at interface of biofilm and fluid is 40% of that in liquid and this oxygen content decreases along with the depth of biofilms (Zhang et al., 1995). Since *R. insidiosa* proliferation requires oxygen (Coenye *et al.*, 2003) and *E. coli* O157:H7 is facultative anaerobes, the different respiration properties can be responsible for the stratified distribution of tested species. Moreover, previous results obtained from Biolog identification study (Chapter 4) showed that *R. insidiosa* strain could use acetic acid and formic acid as sole carbon sources. On the other hand, the acetate and formate are the major products from *E. coli* O157:H7 during anaerobic respiration (Clark, 1989; Sauer et al., 1999). Therefore, *E. coli* O157:H7 metabolism may provide energy sources for *R. insidiosa* proliferation. To study this hypothesis, microelectrode technique can be used for oxygen content measurements. For bacteria metabolisms, genomics and proteomics approaches can be powerful tools to identify genetic pathways that critical to dual-species biofilm formation.

Another possible scenario is related with relieved catabolite repression. With absence of catabolite repression, bacteria can use various types of sugars or amino acids as carbon sources. For example the synthesis of β-galactosidase is increased in *E. coli* when glucose is limited in nutrient. This enzyme catalyzes the hydrolysis of disaccharide producing monosaccharide that will be used in bacteria respiration (Huber et al., 1976).

Klebsiella aerogenes can synthesis glutamate and formamide, and ammonia from L-Histidine (Prival and Magasanik, 1971). Hence, it is speculated that one of the tested bacteria might be able to use the organic matters as carbon sources that produced by the other bacterium. In order to verify this idea, spatial distribution of dual-species biofilms formed with single carbon source need to be studied.

Moreover, bacterial motility plays an important role in cell distribution in biofilms. A study of *Pesudomonas aeruginosa* biofilm formation demonstrated that bacteria mutants with motility could climb on top of mushroom-like structures after incubation for 4-day while non-motile cells stayed at the bottom of the structure (Klausen et al., 2003). There are several studies reported a lost of motility in bacteria, such as *E. coli* and *Salmonella* spp., when forming biofilms (Soutourina et al., 1999; Teplitski et al., 2003). Therefore, the effect of motility properties on dual-species biofilm structure needs to be studied.

Besides studying the interaction between tested strains, it is also important to understand the prevalence of *Ralstonia* genus in different fresh-cut processing facilities at various geographic locations as well as differnt seasons. The assessment of the presence of *R. insidiosa* can provide detailed information to justify the sufficiency of current monitoring operations used in fresh produce processing facilities. In addition, foodborne pathogens integrated in dual-species biofilms need to be subjected to various schemes of sanitization treatments in order to improve the efficacy of routine cleaning and disinfection in food processing environments.

Appendix A: Strain identification using 16s rDNA sequencing

Environmental strains used in Chapter 4 were also identified by 16s rDNA sequencing method. The 16S rDNA for each selected strain was amplified by PCR using universal primers designed by Mignard (Mignard and Flandrois, 2006). The 16S rDNA sequences were determined at University of Maryland Institute for Bioscience & Biotechnology Research and used as query sequences to search NCBI databases for species matches using the online Blast tools (NCBI, BLAST). The 16s rDNA sequencing results were listed in the table below. Of the 13 strains used in this study, the species of 5 were confirmed using 16S rDNA sequences. Another 6 were found to be of different species, but the same genus as previously identified. For the rest two isolates, 16S rDNA sequences placed one of them into closely related genus to the ID result by Biolog III and only one strain was identified as unrelated genus in comparison to Biolog ID result.

Table A. List of environmental strains identified by Biolog identification system and 16s rDNA sequencing.

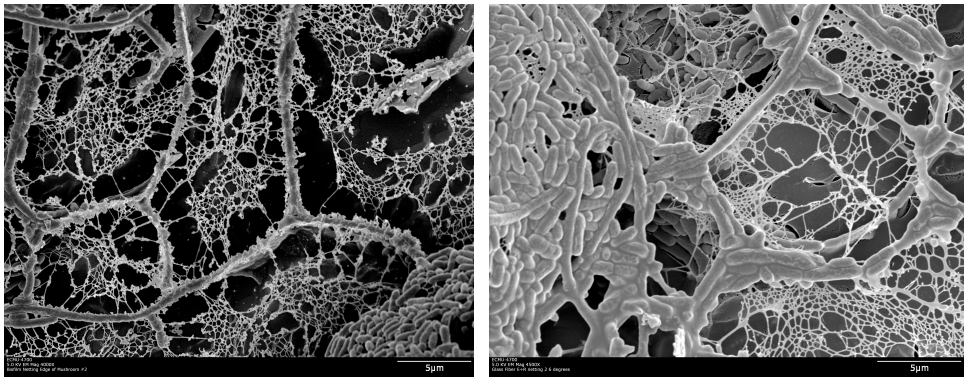
Strain ID	Biolog ID [a]	16s rDNA sequencing
FC2203	***Acinetobacter*** *genospecies 3*	***Acinetobacter*** *baumannii* [b](100%)
FC1169	***Enterobacter cloacae***	***Enterobacter cloacae*** (99%)
FC2134	*Flavimonas oryzihabitans* ***Pseudomonas*** *oryzihabitans*	***Pseudomonas*** *mendocina* (99%); ***Pseudomonas*** *aeruginosa* (99%)
FC2202	***Klebsiella pneumoniae***	***Klebsiella*** *variicola* (100%); ***Klebsiella pneumoniae*** (100%)
FC1161	***Pantoea*** *agglomerans*	***Pantoea*** *ananatis* (99%)
FC3223	*Pectobacterium cypripedii*	*Paenibacillus* sp. (99%)
FC1136	***Pseudomonas fluorescens***	***Pseudomonas fluorescens*** (99%)
FC2208	***Rahnella aquatilis***	***Rahnella aquatilis*** (100%)
FC2204	***Stenotrophomonas*** *rhizophila*	***Stenotrophomonas*** *maltophilia* 100%)
FC1167	*Burkholderia caryophylli*	*Ralstonia solanacearum* (99%)
FC1138	***Ralstonia*** *insidiosa*	***Ralstonia*** *solanacearum* (100%); ***Ralstonia*** *pickettii* (100%)
FC1140	***Rhizobium radiobacter***	***Rhizobium radiobacter*** (100%)
FC1224	***Flavobacterium*** *resinovorum*	***Flavobacterium*** *columnare* (100%)

[a] Species assignment was based on species ID acquired using Biolog GenIII bacterial ID system (Liu *et al.*, 2013). Genus or species name in bold case indicates match between Biolog III and rDNA sequencing.

[b] *Acinetobacter baumannii* belonged to *Acinetobacter* genospecies 2 group.

Appendix B: Morphology of biofilms under scanning electronic microscope

Morphology of monoculture *R. insidiosa* and dual-species biofilms grown on glass fiber filter paper were observed using Low-temperature Scanning Electron Microscope (SEM). Observations were performed using an S-4700 field emission scanning electron microscope (Hitachi High Technologies America, Inc., Pleasanton, CA) equipped with a Quorum PP2000 (Quorum Technologies, East Sussex, UK) cryotransfer system. Biofilms grown on glass fiber was excised and placed on flat specimen holders consisting of 16x30mm copper plates that contained a thin layer of Tissue Tek (OCT Compound, Ted Pella, Inc., Redding, CA), which acted as the cryo-adhesive upon freezing. The samples were frozen conductively and transferred to a LN_2 Dewar for cryotransferred under vacuum to the cold stage in the prep-chamber of the cryotransfer system. The specimens were 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 etching, the temperature inside the chamber was lowered below -130°C, and the specimens were coated with a 10nm layer of platinum. An accelerating voltage of 5kV 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.



(A) (B)

Figure B SEM images of mono and mixed-culture biofilm formed by *R. insidiosa* and dual-species biofilms with *E. coli* O157:H7 (FS4052). (A) Monoculture 3 d *R. insidiosa* biofilm; (B) 3 d dual-species biofilms.

Appendix C: *R. insidiosa* aggregates formed in suspension

The aggregation within *R. insidiosa* suspension was examined followed the same procedure described in Chapter 6. The aggregates were observed under fluorescence microscope by taking Z-stack images. There were large aggregates formed among *R. insidiosa* cells, while planktonic cells still observed in the suspension.

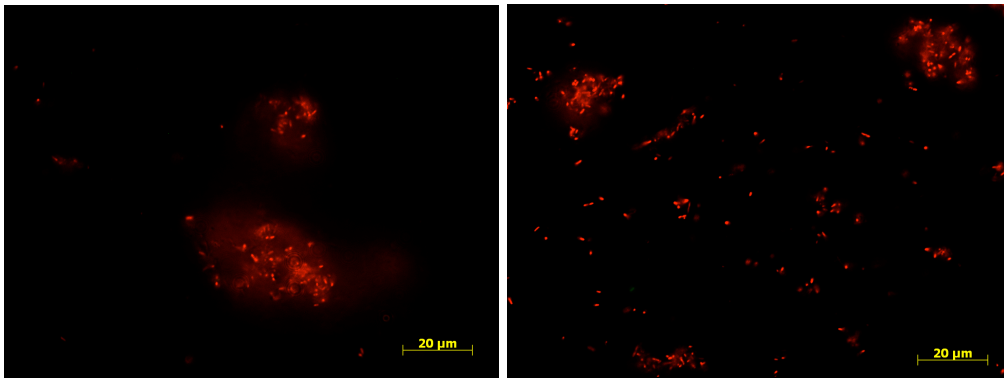


Figure C. Aggregates formed by *R. insidiosa* at room temperature after static culturing in sterile PBS for 24 hours.

Appendix D: Dual-species biofilms formed by *R. insidiosa* and other environmental strains

The total biomass of dual-species biofilms formed by *R. insidiosa* and two environmental strains isolated from fresh-cut processing facilities were measured and compared with the respective monoculture biofilms. The monoculture and dual-species biofilms were formed on 12 well tissue culture plates and the same staining and measuring methods were used as described in Chapter 4, 5. Results acquired from the experiment showed that there was a slight increase of total biomass in dual-species biofilms in comparison to the monoculture *R. insidiosa* biofilm, whereas this increase was not significant.

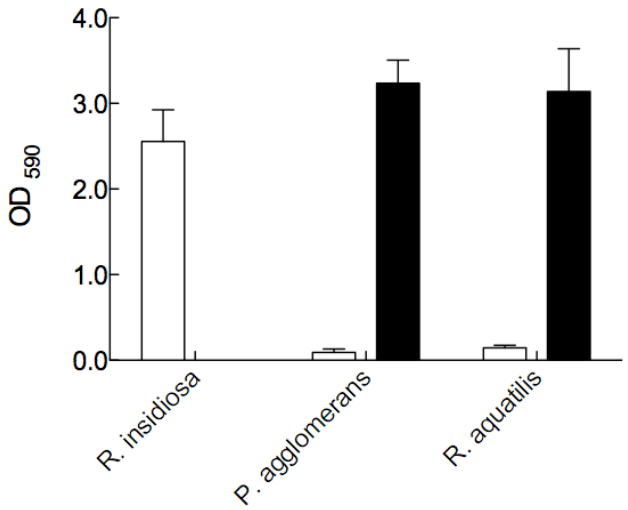


Figure D. Total biomass production of the monoculture (▭) and dual-species biofilms (■) formed by environmental strains with or without *R. insidiosa*.

Appendix E: Total biomass production of 1-day *R. insidiosa* biofilm grown on glass slide before and after heat treatment

In order to understand the heat treatment that employed in the present study, remained biomass was measured by using crystal violet staining assay that described in Chapter 5 with modifications. Briefly, the 1-day *R. insidiosa* biofilm was submerged in 3 ml crystal violet dye for 15 mins. Then the sample was rinsed off thoroughly with tap water and the dye was dissolved in 3 ml 33% acetic acid solution. The optical density at 590 nm was measured. Result acquired from this study showed a significant reduction of the total biomass in *R. insidiosa* biofilm after the heat treatment in comparison with the untreated control. On the other hand, the high OD value of the heat-inactivated biofilm suggested that the major biofilm structure remained after the heat treatment.

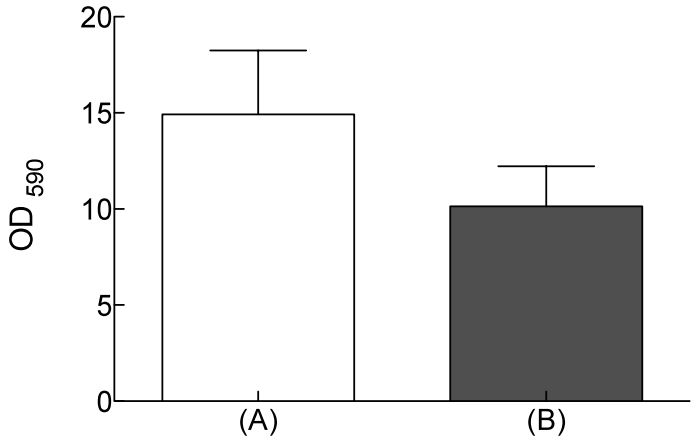


Figure E. Total biomass production of 1-day *R. insidiosa* biofilm formed on glass slide in drip flow biofilm reactor before heat treatment (A) and after heat treatment (B). Serial dilution was made to keep the optical density reading between 0.2-0.8.

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