



Ralstonia insidiosa induces cell aggregation of *Listeria monocytogenes*



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ABSTRACT

Biofilm formation is an important strategy for foodborne bacterial pathogens to survive in stressful environments such as fresh produce processing facilities. Bacterial cell aggregation strongly promotes the initiation of microcolonies and the formation of biofilms on abiological surfaces. We previously showed that *Ralstonia insidiosa*, an environmental bacterial species frequently isolated from fresh produce facilities, may serve as a “bridge bacterium” that strongly enhanced the incorporation of several foodborne bacterial pathogens into dual species biofilms. While the *R. insidiosa* strain exhibited moderate cell aggregation in liquid culture, co-culturing *Listeria monocytogenes* with *R. insidiosa* resulted in significant augmentation of cell aggregation. Electron microscopy indicated that *L. monocytogenes* cells were initially attracted to the *R. insidiosa* aggregates and formed large dual species aggregates that were predominately composed of *L. monocytogenes* cells. The predominant presence of *L. monocytogenes* in the dual species aggregates was also confirmed by differential plating. These findings suggest that bridge bacteria such as *R. insidiosa* play critical roles in the survival of foodborne bacterial pathogens, such as *L. monocytogenes* and *Escherichia coli*, by promoting multispecies biofilm formation. The implications of such bridge bacteria on food safety need to be further evaluated.

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1. Introduction

Listeria monocytogenes was first recognized as a foodborne pathogen subsequent to a series of listeriosis outbreaks implicating various types of foods in the 1980s (Farber & Peterkin, 1991). Over the last few decades, *L. monocytogenes* continued to be a major public health threat, especially involving ready-to-eat foods (RTEs), including fresh produce. A recent large scale outbreaks which caused 147 infections in 28 states and at least 33 deaths, implicated cantaloupes grown and packed in one farm in Colorado (CDC, 2012; McCollum et al., 2013).

L. monocytogenes strains are widely carried by domestic and wild animals, as well as by asymptomatic humans (Lyautey et al., 2007a, 2007b; Valderrama & Cutter, 2012). Besides animal hosts,

they are readily isolated from soil, water, and other environmental matrices (Stea, Purdue, Jamieson, Yost, & Hansen, 2015). Animal originated raw foods and processing premises are often contaminated with *L. monocytogenes* (Carpentier & Cerf, 2011). Recent surveys of RTE foods showed great variation of *L. monocytogenes* prevalence among types of RTEs and their origins (EFSA, 2013; Little, Sagoo, Gillespie, Grant, & McLaughlin, 2009; Pouillot et al., 2015). The presence of *L. monocytogenes* on fresh produce and in fresh-cut produce processing environments (Cartwright et al., 2013; Little et al., 2007) is especially alarming, because of the lack of an effective kill step for pathogen inactivation during fresh-cut processing or food preparation by consumers.

L. monocytogenes has been shown capable of long term survival in environmental and food matrices (Bruno & Freitag, 2011; Locatelli, Spor, Jolivet, Piveteau, & Hartmann, 2013; Wen, Karthikeyan, Hawkins, Anantheswaran, & Knabel, 2013). In addition, *L. monocytogenes* is capable of growing at refrigeration temperature (Huang, Luo, & Nou, 2015; Walker, Archer, & Banks, 1990) and has good tolerance to acidic, alkaline, and high salt media (Liu, Lawrence, Ainsworth, & Austin, 2005), suggesting that

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L. monocytogenes has evolved to gain fitness of surviving in environments independent of animal hosts. Biofilm formation may play critical roles in the survival of *L. monocytogenes* in the environmental and food matrices. Formation of multispecies biofilm communities is of central importance for the survival of microorganisms in the stressful environments outside animal hosts. Indeed, it was estimated that over 90% of bacterial cells existed in the form of polymicrobial biofilm communities (Petrova & Sauer, 2012).

Ralstonia insidiosa is a bacterium, along with closely related and better known species *Ralstonia picketti*, widely present in aqueous environments including municipal water or even medical water purification systems and is well adapted for growth in nutrient deficient environments (Coenye, Goris, De Vos, Vandamme, & LiPuma, 2003; Ryan & Adley, 2014; Ryan, Pembroke, & Adley, 2011). In our previous study, *R. insidiosa* was over-represented among environmental bacterial isolates from fresh produce processing facilities with strong biofilm formation potentials (Liu et al., 2013). We previously showed that *R. insidiosa* strongly promoted the incorporation of *Escherichia coli* O157:H7 cells into dual species biofilms (Liu, Nou, Lefcourt, Shelton, & Lo, 2014, 2015). In most cases, co-culturing of *E. coli*, *Salmonella enterica*, or *L. monocytogenes* strains with *R. insidiosa* resulted in biofilm formation where the total biomass was significantly greater than the sum of that in monocultural biofilms (Liu et al., 2016), indicating a synergistic interaction between *R. insidiosa* and other bacterial strains in biofilm formation. This observation suggests that *R. insidiosa* plays an important role in multispecies biofilm formation in the environments, such as that in the facilities of fresh produce processing. The mechanism of this synergistic interaction is yet to be demonstrated, nevertheless, this process seems to involve direct cell–cell interactions (Liu et al., 2016).

Cell aggregation is an important mechanism for bacteria settling on abiotic surfaces and initiation of microcolonies. Some strains of *L. monocytogenes* autoaggregate in liquid culture. For these strain, deletion of the key virulence regulator gene *prfA* or the gene *actA*, which is controlled by *prfA*, abolished the autoaggregation in vitro and resulted in reduced persistence in gut lumen and diminished shedding in feces (Travier et al., 2013). Therefore, ActA-dependent cell aggregation is both important for biofilm formation in vitro and for persistence in host animals. Inactivation of *secA2*, a non-essential paralogue of *secA*, or two SecA2-dependent cell wall hydrolases gene *cwhA* and *murA*, promoted extensive cell aggregation, sedimentation, and formation of a filamentous biofilm with aerial structures (Renier et al., 2014). Here we demonstrate that *R. insidiosa* induces cell aggregation by *L. monocytogenes*, which provided a mechanism for enhanced biofilm formation and surface colonization by foodborne pathogens in a polymicrobial community.

2. Material and methods

2.1. Bacterial strains and growth media

R. insidiosa strain FC1138 was isolated from food contact surface in a fresh-cut processing facility after routine sanitization (Liu et al., 2013). *R. insidiosa* is a relatively slow grower at the conditions used in the laboratory and the optimal growth conditions have not been determined. Three *L. monocytogenes* strains were used in this study. ATCC 13932 is serotype 4b and characterized as a lineage III strain. Both strains NRRL B-57616 (1/2b, lineage I) and NRRL B-57617 (1/2a, lineage II) were isolated from the 2011 cantaloupe associated outbreaks in Colorado. These strains were obtained through the ARS Culture (NRRL) Collections at ARS National Center for Agricultural Utilization Research, Peoria, IL. (Courtesy of Dr. T. Ward). Tryptic soy broth (TSB, BD Biosciences, San Jose, CA) and tryptic soy agar (TSA,

BD Biosciences) were used for routine culturing of both *R. insidiosa* and *L. monocytogenes* strains. Diluted TSB (10%) was used for culturing bacterial cells in cell aggregation assays. TSA supplemented with 1 mg/L of β -glu (5-Bromo-4-chloro-3-indolyl- β -D-glucopyranoside, Chem-Impex Int'l Inc., Wood Dale, USA) was used for the enumeration of *R. insidiosa* and *L. monocytogenes*. On this non-selective agar plate, *R. insidiosa* forms characteristic elevated small white colonies, and *L. monocytogenes* blue colonies due to its β -glucosidase activity.

2.2. Growth kinetics in liquid media

R. insidiosa and *L. monocytogenes* strains, individually and in combination, were grown in 200 μ l TSB and 10% TSB in 96-well microplates at 30 °C with shaking. A Synergy 4 Hybrid microplate reader controlled by Gen5 Software (BioTek Instruments, Winooski, VT) was used for microplate incubation and for obtaining bacterial growth kinetics information by measuring OD₆₀₀ at 30 min intervals during the incubation. Data were analyzed using OriginPro 7.5 (OriginLab Corporations, Northampton, MA).

2.3. Cell aggregation

Single colonies of *R. insidiosa* and individual *L. monocytogenes* strains were inoculated into TSB and incubated at 30 °C for 24 (LM) to 48 (RI) hours, with moderate aeration (200 RPM orbital shaking). Cells were harvested by centrifugation and resuspended in 10% TSB, and the cell density was adjusted to OD₆₀₀ = 0.1 (~10⁸ cells/ml) by further diluting in 10% TSB. These inoculums (10 ml in 10% TSB) were either mixed with 10 ml 10% TSB (for monoculture) or combined 1:1 (for dual-species culture), and incubated in 90 mm polyethylene petri dishes at 30 °C with low speed orbital shaking (60 RPM) for up to 5 days. The progression of cell aggregation in the petri dishes was monitored by daily visual inspections.

2.4. Aggregate quantification and cell enumeration

After incubation, the content of each petri dish was transferred into a 50 ml Falcon tube. Each culture was centrifuged at low speed (800 rpm in a Thermo Scientific legend \times 1R centrifuge) for 5 min to precipitate cell aggregates. Supernatant was removed and plated on β -glu supplemented TSA plates after adequate 10-fold dilutions. The pellets were washed twice with phosphate buffered saline (PBS pH7.2, Fisher Scientific, Fair Lawn, NJ), resuspended in 10 ml of PBS, and vortexed for 15 min to disperse cells in the aggregates. The cell suspension was similarly diluted and plated on β -glu supplemented TSA plates. All plates were incubated at 30 °C for 48 h. Then the blue *L. monocytogenes* and white *R. insidiosa* colonies were separately enumerated.

2.5. Electron microscopy

Cell suspensions from petri dishes with visible cell aggregates were removed with minimal perturbation to the aggregates. The granules or flocks of cell aggregates were then transferred to the surface of small pieces of nitrocellulose filter paper.

For transmission electron microscopy (TEM), cells in the aggregates were immediately fixed by submerging the loaded filter paper in freshly made 2.5% glutaraldehyde, 0.05 M NaCacodylate, 0.005 M CaCl₂ (pH 7.0) for 2 h, then refrigerated at 4 °C overnight. After multiple buffer rinses they were further post-fixed in 1% buffered osmium tetroxide for 2 h. Sample dehydration, embedding, sectioning, staining, and microscopic examination were carried out as previously described (Liu et al., 2014).

2.6. Statistic analyses

Bacterial cell enumeration for the cell suspensions and cell aggregates were conducted in three replicates. For each species, the percentage of cell counts in the aggregates was independently calculated. Tukey's test was performed for statistical analysis using IBM SPSS software version 19.0. Data were presented as the mean values \pm SD ($n = 3$). Differences were considered significant when $p < 0.05$.

3. Results

3.1. Cell aggregation in liquid cultures

It was previously shown that co-culturing with *R. insidiosus* significantly increased the cell counts of *L. monocytogenes* as well as total biomass in biofilms (Liu et al., 2016). To further characterize the interactions between these two bacterial species, they were co-cultured in petri dishes in an effort to scale up biofilm production, where significant cell aggregation was initially observed. In 10% TSB, *L. monocytogenes* strain NRRL B-57616 exhibited limited growth at 30 °C over a period of up to 5 days. Bacterial cells seemed distributed uniformly in the suspension and no trace of cell aggregation was observed. In contrast, *R. insidiosus* strain FC1138 was capable of substantial growth in 10% TSB in the same time period (Table 1). Very fine granules became visible in the petri dishes starting on day 1, and slowly the granules seemed to settle and formed larger aggregates. By day 3, isolated loose aggregates on the bottom of the petri dishes became evident (Fig. 1). In the co-culture of *R. insidiosus* and *L. monocytogenes*, the growth pattern as reflected by the visual observation of turbidity was comparable to that of the *R. insidiosus* monoculture. However, the fine granules appeared earlier than that of *R. insidiosus* monoculture, and more solid structured aggregations were apparent by day 1. By day 2, the cell aggregates in the co-culture plates were seen as consolidated high-density large flocks that were easily separated from the cell suspension.

Two additional *L. monocytogenes* strains, ATCC 13932 and NRRL B-57617 representing two different serotypes than NRRL B-57616, were co-cultured with *R. insidiosus* to determine if the observed aggregation was strain specific. Both of these strains exhibited identical aggregation pattern as that of strain NRRL B-57616 in co-cultures with *R. insidiosus* (data not shown). Therefore, further investigation of the aggregation in the co-culture with *R. insidiosus* was conducted using *L. monocytogenes* strain NRRL B-57616 as a model.

3.2. Growth kinetics of *R. insidiosus* and *L. monocytogenes*

The growth kinetics of *R. insidiosus* and *L. monocytogenes* strain NRRL B-57616, individually or in co-culture, was determined by monitoring the optical absorbance when growing in TSB or 10% diluted TSB (Fig. 2). In TSB, *R. insidiosus* typically exhibited a long lag phase of about 10 h, followed by a precipitous logarithmic growth, with average optical doubling time of 120 min. By approximately 20 h, the optical density reached the apex, which was followed by a steady plateau for the tested period. Under the same conditions, *L. monocytogenes* showed a very brief lag phase. The log phase lasted for about 10 h, with average optical doubling time of 126 min. Its measured optical density was much lower than that of *R. insidiosus* at the apex of growth, as well as at the stationary phase. In contrast, the growth curve of the co-culture did not show the lengthy lag phase due to the growth of *L. monocytogenes*, and reached a growth apex comparable to that of the *R. insidiosus* strain. A noted fluctuation in OD reading was consistently observed around the log phase – stationary phase transition, which was likely caused by cell aggregation and precipitation at this junction. Similar trends but with diminished growth were observed for cultures in 10% TSB.

3.3. Cell distribution in aggregates and suspension

The distribution of *R. insidiosus* and *L. monocytogenes* cells in the culture suspension and in cell aggregates were examined using *R. insidiosus* strain FC1138 and *L. monocytogenes* strain NRRL B-57616 monocultures and co-culture on day 1, 3, and 5 post inoculation (Table 1). In the monoculture of *R. insidiosus*, approximately 3% of cells were found in the cell aggregates at each of the three sampling time. This balance apparently slightly shifted in the *R. insidiosus* – *L. monocytogenes* co-culture, with approximately two fold increase of *R. insidiosus* cells in aggregates. In both monoculture and co-culture with *L. monocytogenes*, the cell counts of *R. insidiosus* noticeably declined on day 5, indicating an accelerated cell death, or cell entering the viable but not culturable (VBNC) state. *L. monocytogenes* was able to grow to approximately 5×10^8 cfu/ml in 10% TSB and formed uniform cell suspension during the course of the experiment. No sign of cell aggregation was observed at any of the sampling points. In the co-culture with *R. insidiosus*, *L. monocytogenes* cells were predominantly located within the aggregates, accounted for 98, 94, and 72% of total *L. monocytogenes* cells in the mixed culture.

Table 1
Distribution of culturable cells in suspension and in cell aggregates.

	<i>R. insidiosus</i>			<i>L. monocytogenes</i> (NRRL B-57616)		
	Suspension (cfu/ml)	Aggregate (cfu)	Ratio ^a (%)	Suspension (log cfu/ml)	Aggregate (log cfu)	Ratio ^a (%)
<i>Monoculture</i>						
Day 0	$5.91 \pm 0.15 \times 10^7$	0	0 ^{ab}	$1.75 \pm 0.25 \times 10^8$	0	0 ^a
Day 1	$2.81 \pm 0.14 \times 10^9$	$2.23 \pm 0.15 \times 10^9$	3.82 ± 0.42 bc	$4.34 \pm 0.60 \times 10^8$	0	0 ^a
Day 3	$2.52 \pm 0.57 \times 10^9$	$1.26 \pm 0.09 \times 10^9$	2.68 ± 0.38 bc	$5.27 \pm 0.40 \times 10^8$	0	0 ^a
Day 5	$1.18 \pm 0.26 \times 10^9$	$6.30 \pm 1.76 \times 10^8$	2.59 ± 0.23 bc	$4.82 \pm 0.21 \times 10^8$	0	0 ^a
<i>Co-culture</i>						
Day 0	$5.91 \pm 0.15 \times 10^7$	0	0 ^a	$1.75 \pm 0.25 \times 10^8$	0	0 ^a
Day 1	$2.46 \pm 0.16 \times 10^9$	$2.79 \pm 0.33 \times 10^9$	5.35 ± 0.42 bc	$4.33 \pm 1.53 \times 10^6$	$3.69 \pm 0.35 \times 10^9$	97.66 ± 0.92^c
Day 3	$3.43 \pm 0.13 \times 10^9$	$2.56 \pm 0.34 \times 10^9$	3.61 ± 0.61 bc	1.00×10^7	$3.24 \pm 0.74 \times 10^9$	94.01 ± 1.29^c
Day 5	$7.47 \pm 2.42 \times 10^8$	$1.32 \pm 0.82 \times 10^9$	8.12 ± 2.64^c	$4.00 \pm 1.00 \times 10^7$	$2.02 \pm 0.66 \times 10^9$	71.51 ± 4.42^b

^a Represents % of cells in aggregates for each species. This value is calculated by dividing the number of aggregate-bound cells by total number of cells in suspension (20 ml) and in aggregates.

^b Values in the same columns follow by superscripts of different letters are significantly different ($p < 0.05$).

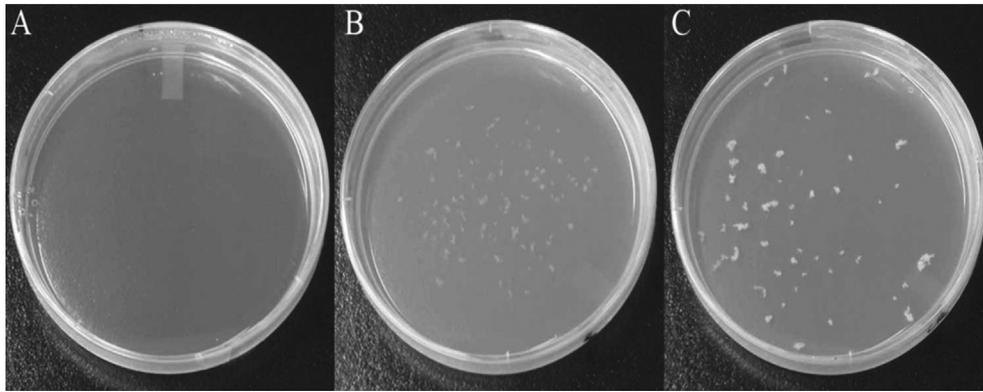


Fig. 1. *L. monocytogenes* (NRRL B-57616) and *R. insidiosa* cell aggregation in mono- and co-cultures. Images taken from 3 day cultures. (A). *L. monocytogenes* monoculture; (B). *R. insidiosa* monoculture; and (C). Co-culture of *L. monocytogenes* and *R. insidiosa*.

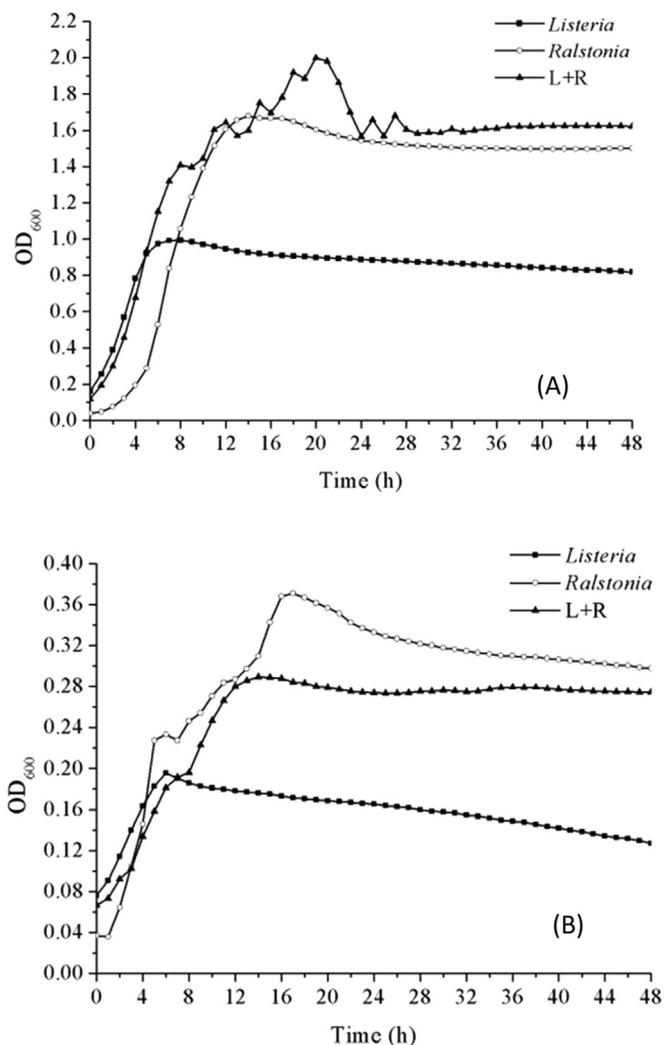


Fig. 2. Growth profiles of *R. insidiosa* and *L. monocytogenes* (NRRL B-57616) in mono- and co-cultures. The growth is presented as OD₆₀₀ measurements over two days. Data is average of 6 measurements in a 96-well microplate. (A). Growth in TSB; (B). Growth in 10% TSB.

3.4. Microscopic examination of cell aggregates

We used TEM to examine the internal structure of the cell

aggregates at early (1 day) and late (5 days) stages. We previously showed that *R. insidiosa* cells could be readily distinguished from *E. coli* O157:H7 cells using TEM (Liu et al., 2014) by virtue of the light staining and the presence of inclusion bodies inside the cells. *L. monocytogenes* cells were also readily distinguishable from those of *R. insidiosa* as they are typically heavily stained, possibly due to the thick cell walls and the absence of outer membrane as Gram⁺ bacterium. At the early stage of cell aggregation, cells in the *R. insidiosa* monoculture aggregates appeared well spaced and there was no sign of cell lysis (image not shown). At the presence of *L. monocytogenes*, cells of both species appeared packed more tightly in the aggregates, often seen with *L. monocytogenes* cells surrounding cores of large and small *R. insidiosa* aggregates (Fig. 3A). Low frequency cell lysis was evident at this stage, as shown by the occasional presence of open and empty “shells” of cells (Fig. 3B). The appearance of these shells was consistent with being derived from cell lysis of *L. monocytogenes* (also see Discussion). Stained substances consistent with the description of extracellular polymeric substances (EPS) were seen mostly surrounding the *R. insidiosa* cells in the cores of the aggregates, and occasional infiltration of the cores by *L. monocytogenes* cells are observed (3C). By day 5, large aggregate cores of *R. insidiosa* were no longer observed, and the aggregates were dominated by *L. monocytogenes* cells (3D).

4. Discussion

L. monocytogenes is capable of long term survival in soil, water, and other environmental matrices. Biofilm formation has been widely recognized as one of the most important strategies for bacteria to survive in stressful environments. Yet laboratory examinations showed that most *L. monocytogenes* isolates had poor to moderate biofilm formation (Borucki, Peppin, White, Loge, & Call, 2003; Harvey, Keenan, & Gilmour, 2007). This apparent paradox indicates that pure culture biofilm formation in laboratory conditions is an inconsistent indicator for the survival of bacteria in the natural environments. Integrated multispecies biofilm communities are the main forms of existence for microorganisms in the nature, where both antagonistic and synergistic interactions drive the progression of the communities. *R. insidiosa*, with strong biofilm formation capability, greatly enhanced the incorporation of foodborne pathogens, including *Listeria monocytogenes*, into dual species biofilms. It seems that *R. insidiosa* could play a role of bridging bacteria (Rickard, Gilbert, High, Kolenbrander, & Handley, 2003) or pioneers in multispecies biofilm formation, and it is likely that such interactions favors the survival of *L. monocytogenes* in

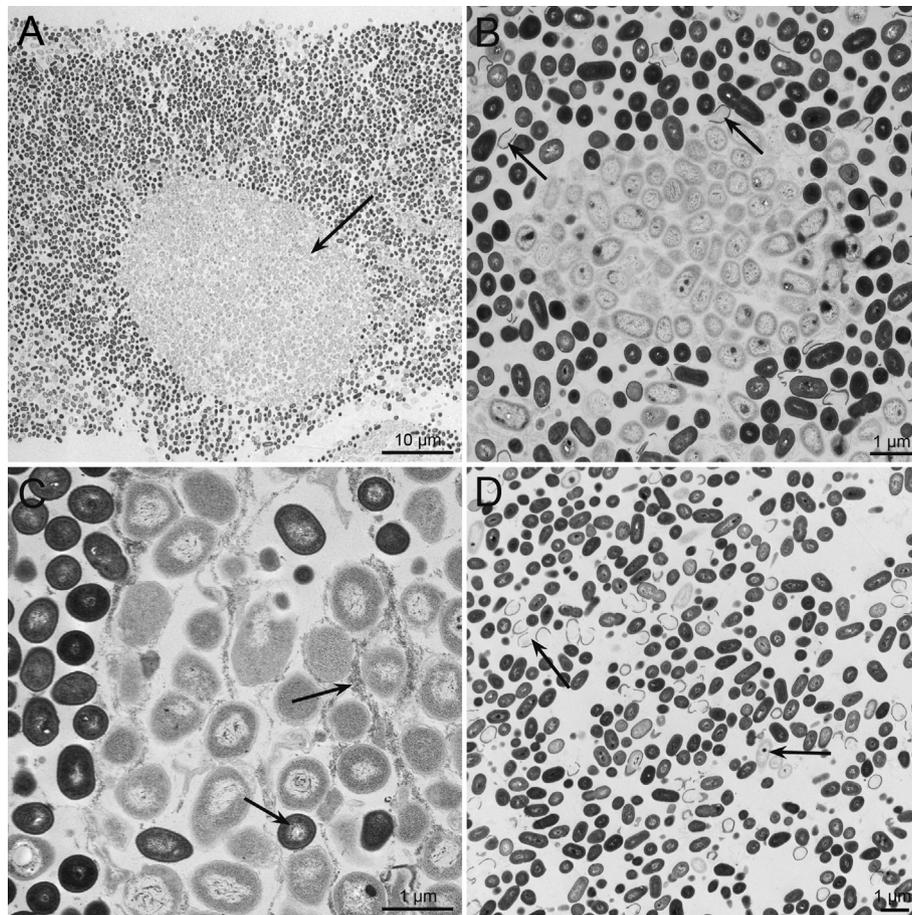


Fig. 3. TEM of *R. insidiosus* and *L. monocytogenes* (NRRL B-57616) co-culture cell aggregation. *R. insidiosus* cells are lightly stained, and *L. monocytogenes* cells are more heavily stained with more clearly defined cell walls. (A). A distant view of cell aggregates showing *L. monocytogenes* cells attracted to a large aggregate of *R. insidiosus* (Arrow) after 24 h co-culturing. (B). A closer view of *L. monocytogenes* surrounding a small aggregate of *R. insidiosus*. Arrows indicating lysed *L. monocytogenes* cells. (C). A very close view of the cell aggregates. Arrows indicate the heavy presence of EPS surrounding *R. insidiosus* cells and the infiltration of *L. monocytogenes* into *R. insidiosus* aggregate. (D). Cell aggregate dominated by *L. monocytogenes* on day 5 of co-culturing. Arrows point to presence of lysed *L. monocytogenes* cells and the infrequent presence of *R. insidiosus* cells.

natural environments (Liu et al., 2016).

In this study we demonstrated that *R. insidiosus* and *L. monocytogenes*, together formed large aggregates, providing a potential mechanism for the synergistic interactions between different species in biofilm formation. Interspecies or intergeneric co-aggregation of bacteria are well documented processes that promote the biofilm formation, especially among the dental plaque microbial communities. Recently multispecies co-aggregation has also been increasingly documented in other microbial communities, including those isolated from fresh water and food processing environments (Rickard et al., 2003). However, there have been very limited reports on foodborne bacterial pathogens co-aggregating with environmental isolates. A majority of *Listeria ivanovii* strains were shown to autoaggregate and to co-aggregate with several reference strains at varying efficiencies (Nyenje, Green, & Ndip, 2012). In this study, we determined that over 90% of the *L. monocytogenes* cells (which did not seem to auto-aggregate) in the co-culture were located inside the cell aggregates, while only a small fraction of cells of the auto-aggregative *R. insidiosus* strain was located in the aggregates. Therefore we refer to this phenomenon as induced aggregation of *L. monocytogenes* by *R. insidiosus*.

However, we have not examined the mechanisms of this interaction. To examine the possibility that the incorporation of *L. monocytogenes* cells in the cell aggregates was achieved by a

mechanism akin to quorum sensing, *R. insidiosus* and *L. monocytogenes* cells were inoculated in Transwell (Corning, NY, USA) composite plate which consisted of two compartments separated by a 0.4 μm pore size polycarbonate membrane that supported free exchange of culture medium and metabolites. When *R. insidiosus* and *L. monocytogenes* strains were incubated in the separate compartments under conditions used in this study, no aggregation by *L. monocytogenes* was observed. We also failed to observe the induced cell aggregation of *L. monocytogenes* when it was cultured in *R. insidiosus* cell free supernatant which should contain all potential diffusible signal molecules produced during the growth of *R. insidiosus* (Data not shown). These outcomes indicated that the induced aggregation of *L. monocytogenes* was dependent on cell to cell contact between cells of the two species, a notion that was consistent with the data of our microscopic examinations. In a recent study, Møretrø and colleagues demonstrated that coaggregation interactions between *Rhodococcus* and *Acinetobacter* strains isolated from food-processing surfaces were not sensitive to heat and Proteinase K treatment, suggesting protein independent aggregation, whereas the coaggregation determinants of the other strains involved proteinaceous cell-surface-associated polymers (Møretrø, Sharifzadeh, Langsrud, Heir, & Rickard, 2015).

Bacterial cell aggregation is an important mechanism of pathogenesis and often involves cell surface structures or adhesins that promote attachment. For example, *E. coli* O104:H4 produce plasmid

encoded aggregative adherence fimbriae (AAF/I) that promote cellular aggregation to form “stacked brick” multilayered biofilm structures on intestinal epithelia (Bielaszewska et al.). In *L. monocytogenes*, cell aggregation has been shown a necessary step in biofilm formation. At least two pathways have been identified to influence cell aggregation in vitro (Renier et al., 2014; Travier et al., 2013). Deletion of the *actA* gene, which is controlled by the major virulence regulator *prfA*, abolished the autoaggregation in vitro by *L. monocytogenes* strains that express an auto-aggregation phenotype (Travier et al., 2013), indicating the expression of *actA* is required for *L. monocytogenes* auto-aggregation. In contrast, inactivation of *secA2*, or two SecA2-dependent cell wall hydrolases gene *cwhA* and *murA*, promoted extensive cell aggregation and sedimentation (Renier et al., 2014), indicating the *secA2* pathway plays an role in preventing *L. monocytogenes* autoaggregation. It is not known if either of these two pathways was activated or inhibited in the *R. insidiosus* induced aggregation.

Although cell aggregation is often considered a promoting factor for biofilm formation, these cell aggregates can also be regarded as a form of biofilms, and may have similar effect for enhancing the survival in adverse environments. High molecular weight extracellular DNA (eDNA) released by fractional cell lysis in a population is of central importance for biofilm formation (Harmsen, Lappann, Knöchel, & Molin, 2010; Shopova et al., 2013). An intriguing observation here is the cell lysis seen by TEM with the co-cultural cell aggregates. Whereas the TEM observation was very consistent with the lysis of *L. monocytogenes* cells based on the morphology of the lysing cells and debris of cell wall structures (Fig. 4), it should be noted that this co-aggregation process was characterized by the decrease of *R. insidiosus* cell counts and the gradual disappearance of large clusters of *R. insidiosus* in the aggregates. Therefore, the interplay of these two species at the population level is a key factor for the formation of dual-species biofilms.

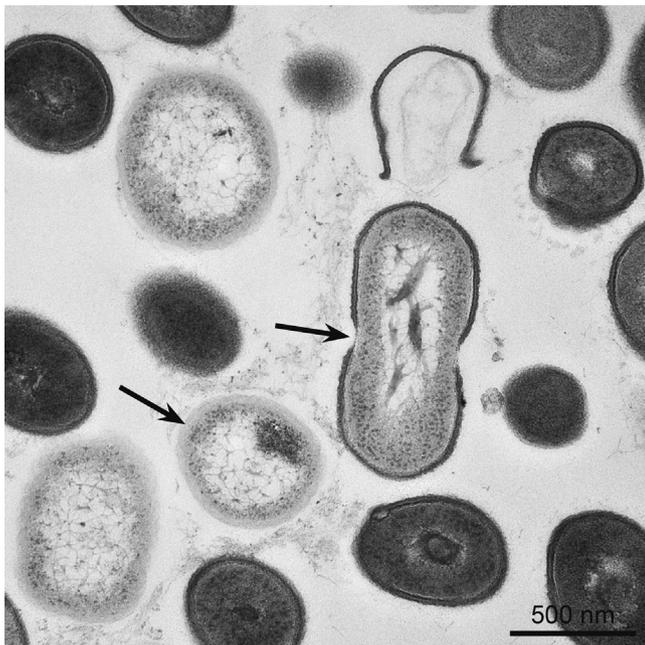


Fig. 4. TEM image showing cell lysis in the aggregate of *R. insidiosus* and *L. monocytogenes* (NRRL B-57616). Arrow on left indicates *R. insidiosus* cell with light staining in comparison to more heavily stained *L. monocytogenes* cells. Arrow on the right points to a cell with gaps in the clearly defined cell wall identical to the heavily stained *L. monocytogenes* cells and the cell wall debris (above). The lighter staining could be due to loss of cytoplasmic contents.

5. Conclusions

In this study we demonstrated that co-culturing an environmental isolate of *R. insidiosus* with *L. monocytogenes* in a low nutrient medium (10% TSB) resulted in enhanced co-aggregation. Over 90% of *L. monocytogenes* cells were incorporated in the aggregates. This study provided a plausible mechanism for the interactions between *R. insidiosus*, a frequent isolate from water and fresh produce processing environments, and foodborne pathogens in forming polymicrobial biofilms.

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