

# A three-tiered approach to differentiate *Listeria monocytogenes* biofilm-forming abilities

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## Abstract

The purpose of this research was to develop a system for cultivating and evaluating *Listeria monocytogenes* biofilms that produces consistent and reliable results. A three-tiered approach was used to evaluate biofilm-forming abilities of three *L. monocytogenes* strains that were originally associated with listeriosis outbreaks. A *L. monocytogenes* ‘honeycomb’ biofilm structure was described. *L. monocytogenes* strains Scott A and V7 were comparable in developing biofilm network structures and F2365 was less effective in forming biofilm. This three-tiered method can be very useful for further biofilm studies.

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**Keywords:** Biofilm development; *Listeria monocytogenes*; Strain variability; Honeycomb structure

## 1. Introduction

*Listeria monocytogenes* is a food-borne pathogen that can lead to potentially life-threatening listeriosis in high-risk populations. Based on the Centers for Disease Control and Prevention’s recent estimations, the annual incidence of death caused by listeriosis is about eight times greater than that caused by *Escherichia coli* O157:H7 [1]. Listeriosis outbreaks have been associated with various processed foods, and the formation of *L. monocytogenes* biofilms in the processing environment could be an important source of secondary contamination [2–5]. To properly evaluate the risks associated with *L. monocytogenes* and to develop effective strategies to minimize listeriosis incidences, advanced knowledge of *L. monocytogenes* biofilm is greatly needed.

It is established that *L. monocytogenes* strains vary in their abilities to cause disease [6–9], and certain *L. mono-*

*cytogenes* strains involved in outbreaks have been repeatedly isolated from the food-processing plant environment [10]. To examine the possible connection between biofilm-forming capabilities and virulence in *L. monocytogenes*, Djordjevic et al. [11] conducted a study using a rapid microtiter plate assay. They reported that the mean biofilm production of lineage I *L. monocytogenes* strains, which contained a majority of strains from human listeriosis cases, was significantly greater than those observed for lineage II and III strains. These data, which are based on attachment measured by crystal violet staining, suggest a potential correlation between biofilm-forming strains and virulent isolates causing listeriosis. In another study, Kalmokoff et al. [12] used scanning electron microscopy (SEM) to examine biofilm formation among 36 *L. monocytogenes* strains, most of which were isolated from outbreaks, covering serotypes 1/2a, 1/2b, 1/2c, 4b, and 4c. Under their screening conditions, the authors found that only one *L. monocytogenes* strain formed biofilm, while all the other isolates, including Scott A [13], only adhered as single cells to the surfaces. However, Chae and Schraft [14] evaluated the adherence of 13 *L. monocytogenes* strains for up to 4 days. The adhered cells were scraped from surfaces and the number of biofilm cells was measured by plate counting. They concluded that all investigated strains, including Scott A, formed biofilms.

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To clarify these diverse results, more consistent *L. monocytogenes* biofilm cultivation and evaluation methods are needed. Communication of reliable experimental procedures and sharing of observation details are very important in promoting research in this area. Since accurate differentiation of biofilm-forming capabilities among *L. monocytogenes* strains is essential for proper assessment of the correlation between strain virulence and niche fitness, biofilm architecture needs to be examined in more *L. monocytogenes* strains.

The objective of this study was to properly assess the variability in biofilm-forming capabilities among *L. monocytogenes* strains. A three-tiered approach is presented to differentiate strains' abilities to attach, to form complex cell attachment patterns, and to progressively develop three-dimensional biofilm architecture. Three *L. monocytogenes* isolates that were originally associated with listeriosis outbreaks were evaluated in this study.

## 2. Materials and methods

### 2.1. Bacterial strains

Three *L. monocytogenes* strains were used in this study: Scott A (serotype 4b, clinical isolate), V7 (serotype 1/2a, milk isolate) [15], and F2365 (serotype 4b, associated with the 1985 listeriosis outbreak in California) [16]. All *L. monocytogenes* strains were maintained in tryptic soy broth (TSB) (Becton Dickinson and Company, Sparks, MD, USA) with 20% glycerol at  $-80^{\circ}\text{C}$ . Cells were sub-cultured by inoculating TSB with 1% of the frozen culture and incubating for 18 h at  $35^{\circ}\text{C}$  ( $\text{OD}_{600\text{nm}}$  0.5–0.7). For microscopic studies, 1–2% of the above cultures were inoculated into fresh TSB and incubated for 18 h at  $35^{\circ}\text{C}$  ( $\text{OD}_{600\text{nm}}$  0.5–0.7).

### 2.2. Rapid attachment assay by polystyrene culture tube–crystal violet staining method

A rapid tube adherence test [17] was used with modification. One percent of an overnight *L. monocytogenes* culture was inoculated into 2 ml TSB in polystyrene culture tubes (Fisher Scientific, Pittsburgh, PA, USA) and incubated at  $37^{\circ}\text{C}$  for 24 h or 72 h. After incubation, 500  $\mu\text{l}$  0.1% crystal violet (Fisher Scientific) were added to each culture tube and incubated at room temperature for 15 min. Culture tubes were rinsed three times with 4 ml distilled water to remove unattached cells and residual dye. Crystal violet-stained attached cells were de-stained with 4 ml 95% ethanol. Attachment capabilities of *L. monocytogenes* strains were measured by the absorbance at 600 nm using a Spectronic 1201 spectrophotometer (Milton Roy Company, Ivyland, PA, USA). Each tube test was performed in triplicate.

### 2.3. Assessment of cell attachment patterns by wide-field fluorescence microscopy (WFM)

WFM was used to screen for *L. monocytogenes* strains' biofilm-forming potentials on Lab-Tek Permanox plastic chamber slides for optimal cell performance (Nalgene Nunc International, Naperville, IL, USA). Chamber slides were inoculated with a mixture of 500  $\mu\text{l}$  overnight *L. monocytogenes* culture and 500  $\mu\text{l}$  fresh TSB. A chamber with 1 ml TSB was used as a control. Inoculated slides were incubated at  $35^{\circ}\text{C}$  for up to 72 h. At the appropriate time points, samples were removed from the incubator. Unattached cells and culture medium were washed off using 30 ml phosphate-buffered saline (8 g NaCl, 0.2 g KCl, 1.44 g  $\text{Na}_2\text{HPO}_4$ , 0.24 g  $\text{KH}_2\text{PO}_4$  per liter, pH 7.4). Surface-attached cells were fixed with 1% formaldehyde in phosphate-buffered saline for 30 min at  $4^{\circ}\text{C}$  and then stained with 0.01% acridine orange (Sigma, St. Louis, MO, USA) for 10 min at room temperature. The acridine orange was poured off and excess stain was removed by washing the slides three times with 30 ml phosphate-buffered saline. Samples were never allowed to dry, and phosphate-buffered saline was used for mounting coverslips on the slides.

Samples were observed using a Zeiss Axioskop microscope fitted with a Zeiss AttoArc, HBO 100-W mercury lamp (Carl Zeiss, Oberkochen, Germany). The FITC filter was used to observe the attachment patterns with excitation wavelength of 450–490 nm. For each sample, digital pictures were captured at low magnification to obtain an overview of the pattern of attached cells after sample processing as well as at high magnification to observe details of the two-dimensional pattern and individual cells.

### 2.4. Biofilm observation by SEM

A widely accepted method for the preservation and preparation of biological samples for SEM was used to observe *L. monocytogenes* biofilm structures [12] with modification. Biofilms were cultivated on round stainless steel coupons (12 mm diameter; BioSurface Technologies, Bozeman, MT, USA) for SEM observation. Coupons were cleaned as described previously [12] by boiling in 1% sodium dodecyl sulfate for 5 min, rinsing with 100% isopropanol for 5 min, and washing with distilled water for 5 min. Clean coupons were autoclaved for 20 min at  $121^{\circ}\text{C}$ .

Biofilms were cultivated on sterile stainless steel coupons, each inoculated with an overnight culture plus fresh TSB at a ratio of about 1:2. Inoculated coupons were incubated at  $35^{\circ}\text{C}$  and removed from their containers at selected time intervals.

Upon removal from the culture, coupons were rinsed by gentle repeated immersion in 100 ml fresh TSB for 10 s [12]. Coupons were then placed in glass vials with 2.5%

glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 and fixed at 4°C overnight. The fixing solution was drained and coupons were rinsed in 0.1 M cacodylate buffer. The samples were post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h at room temperature. Post-fixative was drained and coupons were rinsed in 0.1 M cacodylate buffer.

Samples were dehydrated by a graded 100% ethanol: hexamethyldisilazane (Ted Pella, Redding, CA, USA) series (3:1 for 15 min, 1:1 for 15 min, 1:3 for 15 min, 100% hexamethyldisilazane 3×15 min) as opposed to critical point drying which might not cater to the delicate exopolymeric substance (EPS) structures within a biofilm. Samples were left in the last hexamethyldisilazane volume to dehydrate overnight. Dehydrated samples were sputter-coated for 90 s with gold palladium (Pelco Model 3 Sputter Coater, Ted Pella). Samples were observed using a scanning electron microscope (FEI XL30, FEI Company, Hillsboro, OR, USA). Images were captured at low magnification for overview of biofilm structure and high magnification to show details of biofilm architecture. Biofilm cultivations and SEM for each strain at each time point were repeated at least three times.

### 3. Results

#### 3.1. Strain variation in attachment and complex pattern development as determined by WFM

The attachment capabilities of the three *L. monocytogenes* strains were first screened and compared using the polystyrene culture tube assay. *L. monocytogenes* strains Scott A and V7 were found to be comparable in establishing effective attachment to the tube surface, based on their rapid assay absorbance readings. However, the reading for *L. monocytogenes* F2365 was 70–80% of that for Scott A after 3 days of incubation. To further understand the *L. monocytogenes* biofilm differentiation process, we monitored the development of complex patterns of *L. monocytogenes* cells attached to both plastic chamber slides and untreated glass slides by WFM. Comparable results were observed; however, attachment pattern development on untreated glass slides was slower than on plastic chamber slides (data not shown). The pictures represent two-dimensional patterns of attached cells on chamber slides after sample preparation for WFM. A total of at least 58 slides were prepared for up to 13 incubation periods in this

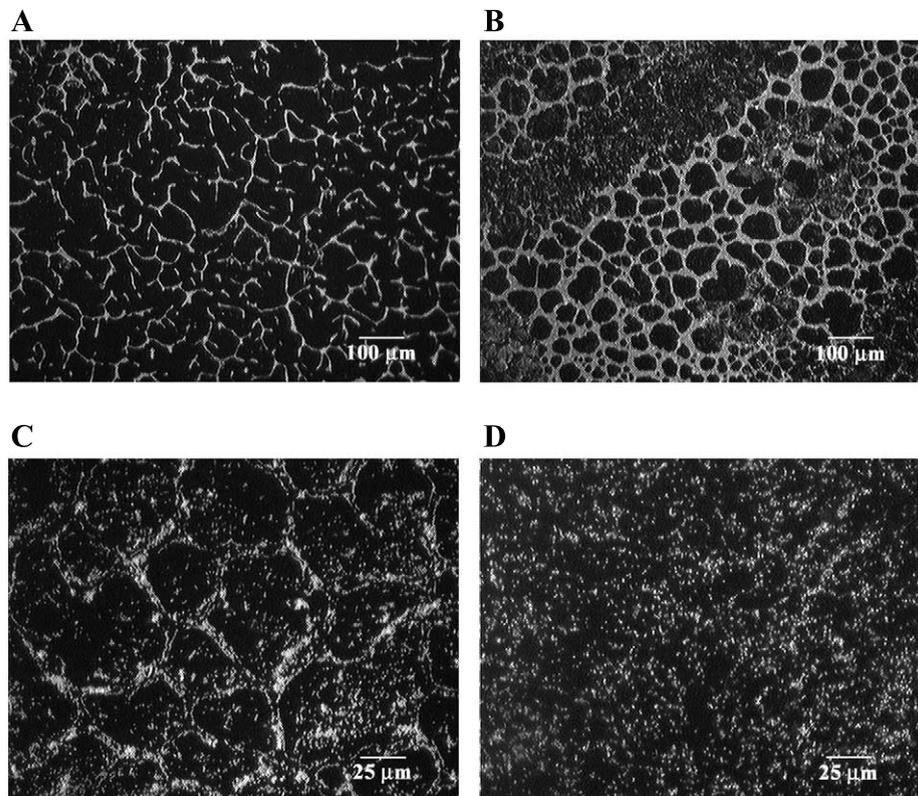


Fig. 1. WFM assessment of complex structure development by *L. monocytogenes* Scott A grown in TSB on plastic chamber slides for 3–72 h, according to patterns observed after sample processing. A: 3 h. B: 6 h. C: 24 h. D: 72 h. At 72 h the net-like pattern is not distinct. Multiple layers may be dense, network may be dissociated, or structure may have peeled from the surface. Pictures of A and B were captured using a 10× objective lens and the bars represent 100 μm; C and D were captured using a 40× objective lens and the bars represent 25 μm.

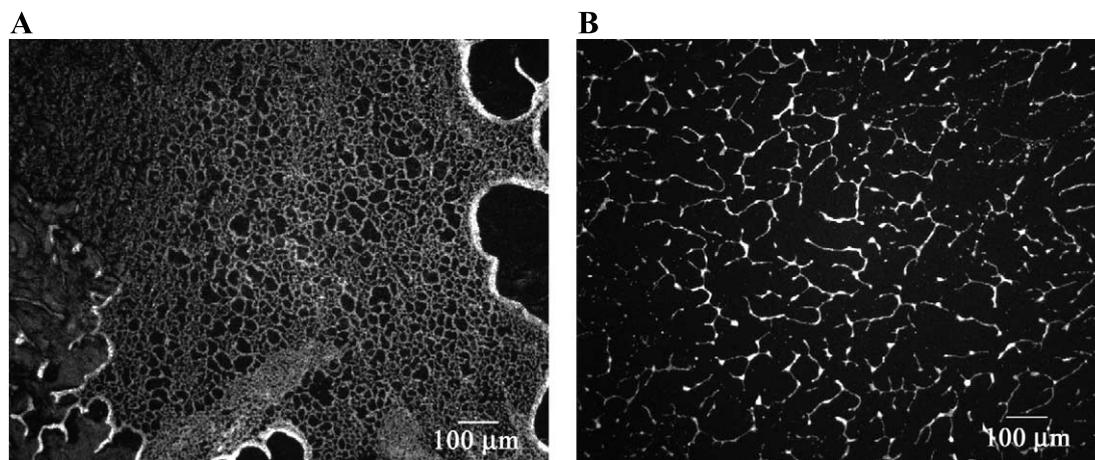


Fig. 2. WFM assessment of structure development by *L. monocytogenes* V7 and F2365 grown in TSB on plastic chamber slides for 24 h, according to patterns observed after sample processing. A: *L. monocytogenes* V7 has established a net-like pattern. Edges of image are representative of film peeling during sample washing. B: *L. monocytogenes* F2365 has not yet established a structure that is observed as a net-like pattern by WFM. Pictures were captured using a 10× objective lens and the bars represent 100 µm.

study. Each of the presented pictures represents an incubation period that was repeated at least three times, and the differences in observed cell pattern complexity among strains remained consistent. We observed attachment and the progression towards an organized net-like pattern by *L. monocytogenes* Scott A cells (Fig. 1). *L. monocytogenes* V7 had very similar complex pattern development as Scott A (Fig. 2A). *L. monocytogenes* F2365 was slower at attaching and forming complex structures, as indicated by its patterns on plastic chamber slides observed by WFM (Fig. 2B). By 24 h, *L. monocytogenes* F2365 established the patterns Scott A established after 3 h of incubation in TSB on plastic chamber slides.

We believe after short incubation when the density of adhered cells was low (Figs. 1A and 2B), the draining of the culture medium and washing process in sample preparation for WFM might slightly alter the true pattern of the adhered cells on the slide. However, at later time points when more cells are firmly attached to the surface and have started to form biofilm, the draining and washing process less likely can affect the structures and the two-dimensional patterns observed by WFM. The image should be closely representative of the structure in the undisturbed culture medium on the slide (Figs. 1B,D and 2A).

Although the development of two-dimensional net-like attachment patterns resembling honeycombs by *L. monocytogenes* strains, as observed by WFM, might have indicated gradual formation of organized biofilm, further illustration with characteristic biofilm architecture details is essential for confirmation.

### 3.2. Differential biofilm development in *L. monocytogenes* strains observed by SEM

Our study based on WFM suggests that *L. monocytogenes* biofilm development probably occurs in several

stages. This observation was further confirmed when the details of biofilm structures were observed by SEM. The *L. monocytogenes* biofilm development and differentiation processes on stainless steel coupons were captured by SEM for Scott A (Fig. 3), V7 and F2365 (Fig. 4). On the stainless steel surface, Scott A first formed microcolonies (Fig. 3A,B). A honeycomb-like structure then developed, containing individual *L. monocytogenes* cells surrounded by EPS (Fig. 3C,D). Three-dimensional structures were very obvious. The Scott A biofilm then became thicker and the three-dimensional structure was more complex (Fig. 3E,F). *L. monocytogenes* V7 shared very similar biofilm architectural features and development with Scott A (Fig. 4A–C). Attachment and biofilm development by F2365 was also observed but seemed to be relatively limited in complexity (Fig. 4D–F).

## 4. Discussion and conclusion

Listeriosis incidence has been associated with various processed foods, and the presence of *L. monocytogenes* in the processing environment is considered to be a serious threat to food safety. Biofilms formed by *L. monocytogenes* and other natural microflora in sanitation-dead corners, such as air or liquid filtration systems, stainless steel surfaces, rubber or Teflon seals, and machine joints, can become the reservoirs for recurring contamination in the food-processing plant environment [4,18]. To simulate these steady-state environments in the food-processing plant, we have examined three *L. monocytogenes* strains for their abilities to develop biofilms in resting containers. Four surfaces were tested though the intentions of this research were not to compare biofilm formation on different surfaces. While searching for the best method for evaluating biofilm-forming capabilities of bacteria, specifically *L. monocytogenes*, we considered usability and efficiency of

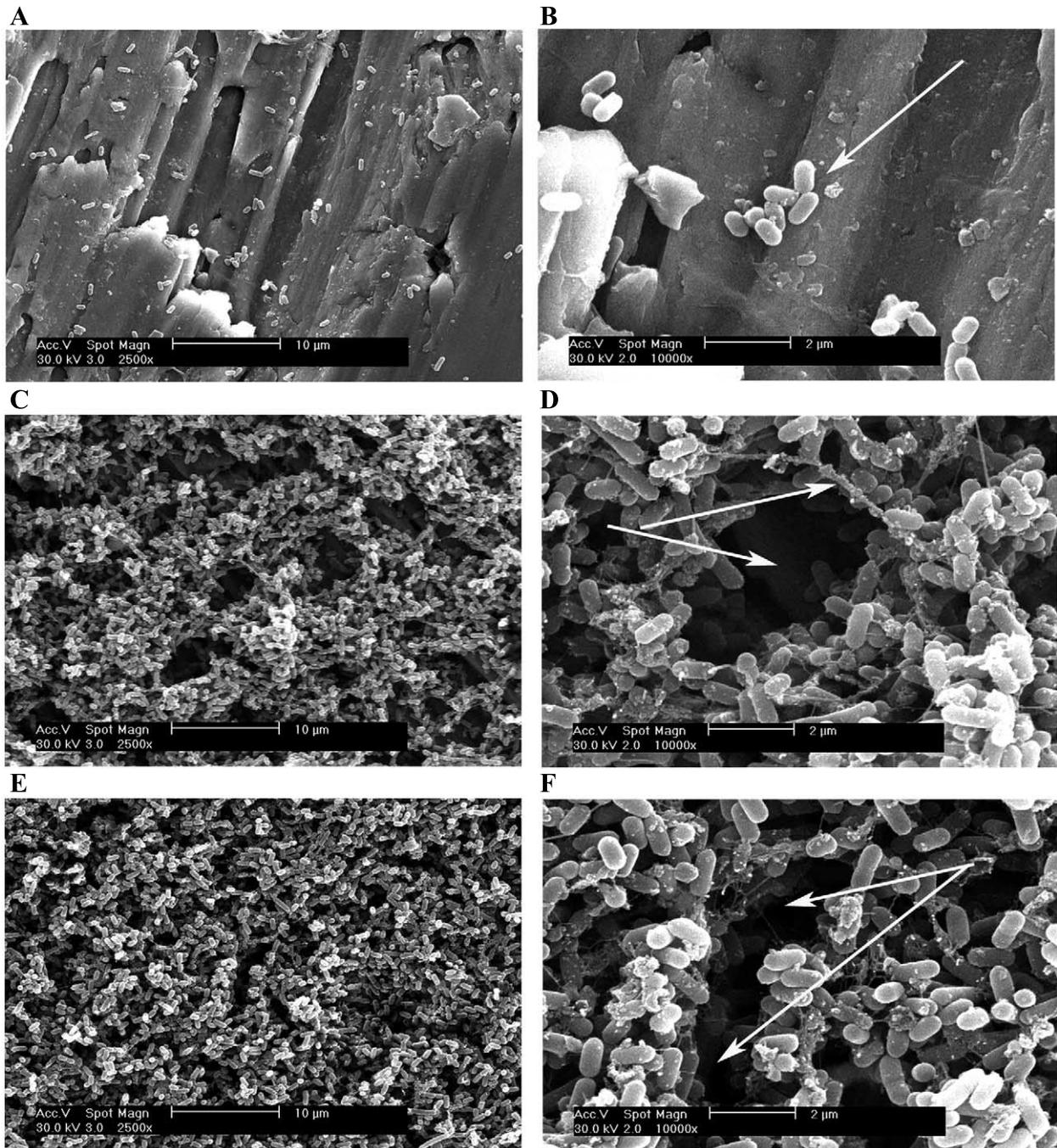


Fig. 3. SEM pictures of *L. monocytogenes* Scott A biofilm development in TSB on stainless steel coupons. A: Overview of 1 h colonization and attachment to the surface. B: Regional magnification of A showing details of structure at 1 h. The arrow indicates the development of a microcolony. C: Overview of the biofilm structure formed at 24 h. D: Regional magnification of C showing details of structure at 24 h. The top arrow points to the EPS webbed between cells. The bottom arrow points to the channel-like structure in the center of a 'honeycomb'. E: Overview of the biofilm structure at 48 h. F: Regional magnification of E at 48 h. The arrows indicate the formation of a three-dimensional channel structure. The bars in A, C, and E represent 10 µm; the bars in B, D, and F represent 2 µm.

the individual methods, including the surfaces used in our study.

A three-tiered approach has been presented for evaluating *L. monocytogenes* strains' abilities to form biofilm. First, attachment capabilities were assessed by the polystyrene culture tube assay. Second, WFM was used to examine the abilities of strains to attach to plastic chamber

slides in complex patterns. The plastic chamber slides were selected because they are easy to manage when dealing with multiple strains, multiple time points, and a fluorescence microscope. Finally SEM was used to verify the formation of *L. monocytogenes* biofilms on stainless steel disks with typical biofilm structure characteristics. Stainless steel was chosen because metal is the best conductor

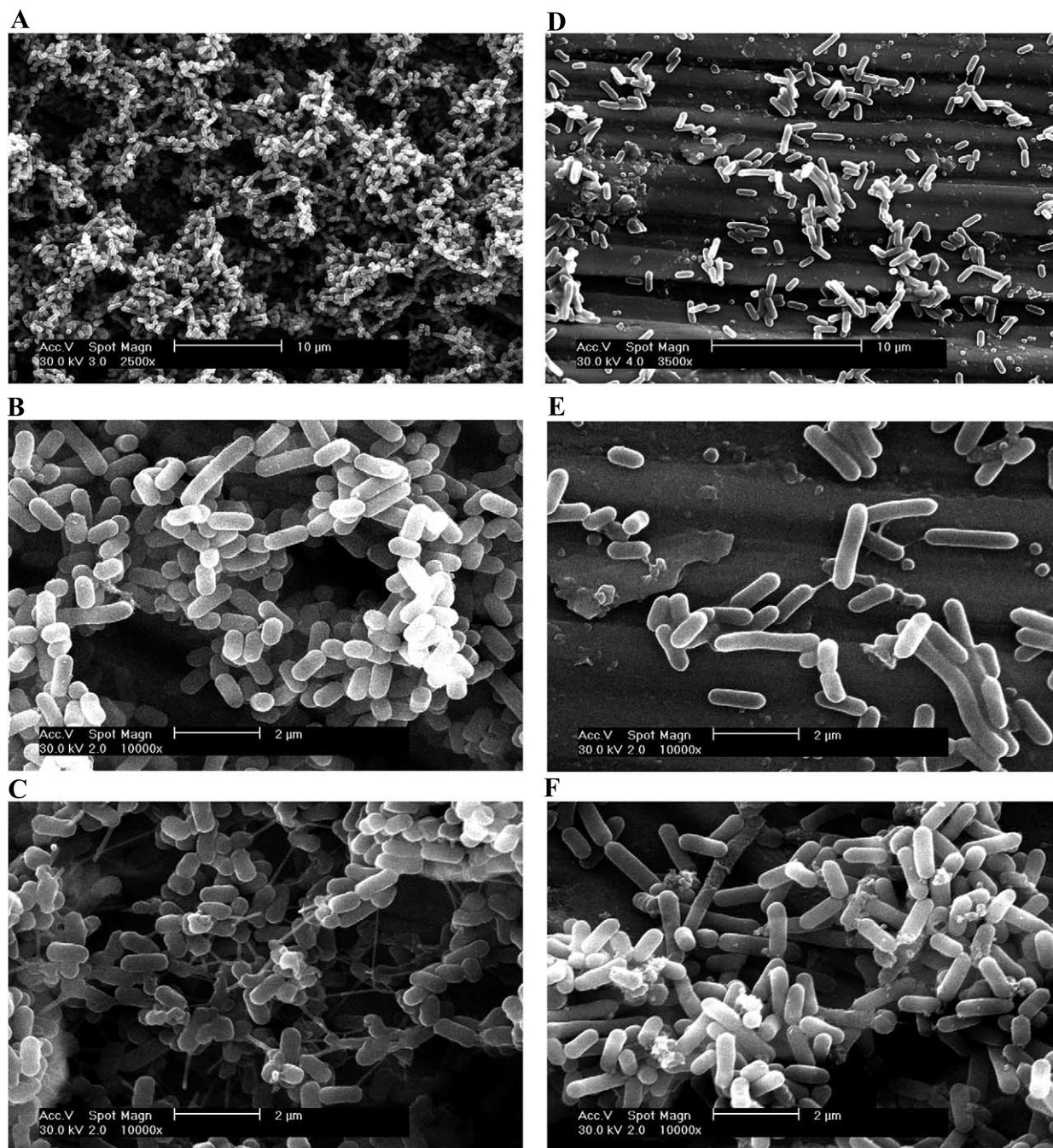


Fig. 4. SEM pictures of *L. monocytogenes* V7 and F2365 biofilms developed in TSB on stainless steel coupons. A: Overview of V7 biofilm at 24 h. B: Regional magnification of A showing details of V7 biofilm at 24 h. C: Regional magnification of V7 biofilm at 48 h (overview not shown). D: Overview of F2365 biofilm at 24 h. E: Regional magnification of D showing details of F2365 biofilm at 24 h. F: Regional magnification of F2365 biofilm at 48 h (overview not shown). The bars in A and D represent 10 µm; the bars in B, C, E, and F represent 2 µm.

for SEM. Results from each of the three methods for the same strain are consistent, confirming that *L. monocytogenes* Scott A forms biofilm at 35–37°C, as reported by Chae and Schraft [14].

To our best knowledge, this is the first time the progressive formation of an organized, net-like attachment pattern by biofilm-forming *L. monocytogenes* strains was captured using WFM. In fact, the WFM pictures presented by Djordjevic et al. [11] to demonstrate *L. mono-*

*cytogenes* biofilm formation on stainless steel and PVC slides were very similar to the pictures we presented at the early stages of net-like pattern development (Fig. 1A).

Using this three-tiered approach, biofilm formation of a *L. monocytogenes* derivative we define as Cal-01, was also evaluated in our laboratory. Neither the branched nor net-like patterns were observed by WFM. Only isolated cells randomly adhered to stainless steel surfaces were observed by SEM. Thus we concluded that there is a strong corre-

lation between the formation of net-like patterns of attached cells by WFM and the complex biofilms observed by SEM in *L. monocytogenes* strains. The less expensive and manageable WFM is more suitable in screening for biofilm-forming strains according to results from this work.

It was observed that the pattern complexity (WFM) and biofilm architecture (SEM) at each particular time point varied slightly among different repetitions, possibly due to growth rate variability and the randomness of the initial event that triggers the differentiation process. In addition, biofilms were not always uniform across a single surface in the same experiment. As demonstrated by SEM, the most developed biofilm structures were usually found in crevices of stainless steel surfaces. We often observed mature biofilm covering a certain percentage of the surface while in other regions the structure was still developing, or the mature structure had detached leaving behind residual EPS. We also observed on occasion the peeling or flaking of *L. monocytogenes* biofilm structures from the surfaces during treatment steps required for both microscopy analyses (Fig. 2A). However, the tendency to form biofilm and the production of EPS within each strain remained consistent regardless of the surface used in the cultivation or the method of evaluation. Further, we have observed that the composition of bacterial culture medium can affect biofilm formation. For instance, TSB is generally a good medium for biofilm cultivation. However, Tween 80 in deMan–Rogosa–Sharpe broth (MRS) significantly inhibits biofilm formation on tested strains including *Lactobacillus* spp. and *L. monocytogenes* (data not shown).

Biofilms by all three *L. monocytogenes* strains share a common structural feature we describe as ‘honeycomb’. Like a honeycomb, the *L. monocytogenes* biofilms were composed of cells stacked upon each other around the perimeter of holes and between holes, with stringy EPS apparently anchoring the cells in their positions. It is likely that the holes throughout the honeycomb-like architecture of these biofilms serve as empty channels for the transportation of water, nutrients and waste. Although *L. monocytogenes* biofilms were previously illustrated in *L. monocytogenes* LO28 [19], to our knowledge this is the first detailed description of *L. monocytogenes* biofilm architecture. The EPS observed in all biofilms of *L. monocytogenes* strains in this study were thread-like, binding one cell to another and to the surface. This biofilm structure is distinctively different from the commonly described ‘mushroom’ biofilm structure, where bacterial cells such as *Staphylococcus* spp. [20] were completely embedded in a thick, gummy EPS. In those cases the amount of EPS synthesized in the biofilm was eye-catching, and its synthesis could be related to strain virulence. *L. monocytogenes* biofilm architecture is probably more similar to that of *Streptococcus mutans* [21] in which a porous, sponge-like structure has been described. Different types of EPS synthesized by bacteria or different signals in-

involved in biofilm development could all contribute to these structural differences.

In conclusion, using the combination of microscopy techniques with multiple time point observations, we are able to comment on *L. monocytogenes* strains’ abilities to form biofilm confidently. Our study provides documentation of biofilm structures by three *L. monocytogenes* strains associated with listeriosis outbreaks. Development of distinct *L. monocytogenes* biofilm architecture is described. Further studies can be conducted to determine the contributions of genetic components and their differential expression to *L. monocytogenes* biofilm development, and to assess the correlation between strain virulence and niche fitness.

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