

**SCANNING ELECTRON MICROSCOPY OF *LISTERIA MONOCYTOGENES* BIOFILMS ON STAINLESS STEEL SURFACES**

MILANOV DUBRAVKA\*, AŠANIN RUŽICA\*\*, VIDIĆ BRANKA\*, KRNJAIĆ D\*\*, PETROVIĆ JELENA\* and SAVIĆ SARA\*

\*Scientific Veterinary Institute "Novi Sad", Novi Sad, Serbia

\*\*University of Belgrade, Faculty of Veterinary Medicine, Serbia

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*Listeria monocytogenes* is the causative agent of numerous epidemics and sporadic cases of illness in humans. Food is the principal route of infection. Raw materials of animal and vegetable origin are the potential sources of contamination with this bacterium, particularly the foodstuff undergoing minimal processing procedures. However, in the recent years, emphasis has been increasingly laid on the importance of post-processing contamination occurring through the contact of products with contaminated surfaces in food-processing plants, where this bacterium can persist for months, even for years, as a biofilm. Although it appears to be widely accepted that *L. monocytogenes* forms biofilms on food-processing surfaces, there has been very little direct microscopical evidence to support this.

In this study we investigated the ability of biofilm formation in 14 strains of *Listeria monocytogenes* of different origin. The biofilms were formed on stainless steel surfaces during 7 days of incubation in a tryptone soy broth supplemented with 0.6% yeast extract (TSB-YE) at a temperature of 25°C. The developed structures were examined using scanning electron microscopy.

Our results revealed significant differences among tested *Listeria monocytogenes* strains in terms of their ability to form a biofilm in given experimental conditions. Seven of 14 investigated strains of *Listeria monocytogenes* did not form a biofilm, and only individual bacterial cells were distributed over the stainless steel surface. The strains classified as biofilm producers formed structures of different appearances, from a uniform, confluent monolayer of bacterial cells to individual large, three-dimensional cell aggregates. All strains proved weak producers of extracellular polymeric substance.

**Key words:** *Listeria monocytogenes*, scanning electron microscopy, biofilm, stainless steel

## INTRODUCTION

*Listeria monocytogenes* is a human pathogen that is responsible for serious infections in immunocompromised individuals and pregnant women. Ingestion of usually heavily contaminated foods is the principal route of infection. The mortality rate in systemic listeriosis has been estimated as between 20% and 40% (McLauchlin *et al.*, 2004). 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 (Mead *et al.*, 1999).

Several studies have focused on elucidating the sources and contamination routes of *L. monocytogenes* in food-processing environments. These studies concluded that raw materials were not a major source of contamination, but that contamination occurred during processing and that the food-processing equipment can act as a reservoir of *L. monocytogenes* (Möretrö and Langsrud, 2004; Vogel *et al.*, 2001). *Listeria monocytogenes* can be found on and in various equipment and in the working environment such as gaskets, conveyor belts, slicing, dicing and packaging machines, containers, knives, tables, drains, floors and walls (Nelson, 1990). Certain clones of *L. monocytogenes* can persist in the food processing environments for years. The possibility that these persistent strains could be specifically adapted to survive in a biofilm is a potential explanation. Biofilm is a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, embedded in a matrix of extracellular polymeric substances that they have produced, and which exhibit an altered phenotype with respect to growth rate and gene transcription (Donlan and Costerton, 2002). Biofilms have the potential to act as a chronic source of microbial contamination, which may compromise food quality and represent a significant health hazard.

Although it appears to be widely accepted that *L. monocytogenes* forms biofilms on food-processing surfaces, there has been very little direct microscopical evidence to support this (Kalmokoff *et al.*, 2001). Scanning electron micrographs demonstrating the presence of individual cells on various surfaces have been reported (Herald and Zottola, 1988; Mafu *et al.*, 1990; Blackman and Frank, 1996). Contrary to that, Chavant *et al.* (2002) and Borucki *et al.* (2003) described the biofilm of some strains of *L. monocytogenes* as a dense three-dimensional cellular network with channels and pores between cell aggregates. Marsh *et al.* (2003) correlated the appearance of the biofilm of *L. monocytogenes* strains with a "honeycomb" pattern. By applying a confocal scanning laser microscopy Chae and Schraft (2000) established a double-layer structure of a *L. monocytogenes* biofilm. The described appearance of the biofilm of *L. monocytogenes* departs from widely accepted "mushroom-shaped" structure, and numerous determining factors are related to experimental conditions, strain characteristics, substrate type, incubation period and temperature, as well as the composition of the nutritive medium.

In this study we investigated the ability of various strains of *L. monocytogenes* to form biofilms on stainless steel surfaces, and the appearance of formed structures was examined by the use of scanning electronic microscopy.

#### MATERIALS AND METHODS

##### *Bacterial strains*

The investigation was conducted using 14 strains of *Listeria monocytogenes*. The strains used in this research were isolated from animals (8 strains), food (4 strains) and feed (1 strain); while a human strain (ATCC 19115) was used as the referent strain. Bacterial strains were identified according to their cultural and tinctorial features, applying catalase, oxidase and CAMP test. Biochemical characteristics were determined applying MICROBACT™ identification system 12L (Oxoid, Basingstoke, UK). Serological typing of the strains was performed using specific sera for O and H antigens for *Listeria* species. (*Listeria antisera*, DENKA SEIKEN, UK). *L. monocytogenes* strain origin and their serological types are displayed in Table 1.

Table 1. *Listeria monocytogenes* strains used in this study

Isolate	Source	Serotype
785/05	brain tissue of sheep	1/2a
593/05	brain tissue of sheep	4b
748/05	feed (hay)	3a
1915/04	brain tissue of sheep	4b
021/04	uterus of cows	3a
A	uterus of cows	3a
C	aborted fetuses of cows	3a
F	uterus of cows	1/2a
16	food (smoked herring)	4b
154	food (frozen salmon)	1/2a
K	rabbit	4b
221	food (smoked salmon)	1/2a
808	food (frozen hake)	1/2b
ATCC 19115	human isolate (control strain)	4b

##### *Preparation of bacterial suspensions*

All strains of *L. monocytogenes* were cultured in a blood agar with 5% sheep blood and incubated at 37°C during 24h. Three to four isolated colonies grown on blood agar were inoculated in 3 mL of tryptone soy broth enriched with 0.6% yeast

extract (TSB-YE) and incubated for 24h at 25°C. After incubation the suspensions were homogenized in the vortex and diluted to 1:40 in a fresh TSB-YE.

The density of the inocula used for biofilm formation was determined by spectrophotometry (UV-VIS spectrophotometer – *Anthelie advanced, Secomam*) and standard plate count method from a series of ten-fold dilutions. Inoculum density was  $OD_{600}=0.098 (\pm 0.015)$ , i.e.  $2-15 \times 10^7$  cfu/mL.

#### *Biofilm formation*

Stainless steel coupons sized 1 cm x 1 cm x 0,2 cm were used as a substrate for biofilm formation. The coupons were boiled for 5 min in a detergent solution and then rinsed five times in distilled water to remove any residual detergent. The coupons were stored in 100% ethanol and sterilized by flaming prior testing. Coupons were then placed into separate wells of a sterile polystyrene 12-well plate (Nunc, Roskilde, Denmark).

Aliquots of suspension 100  $\mu$ L were inoculated onto the stainless steel coupon surface and incubated at 25°C for 3h to allow adhesion to occur. After a 3h incubation, the non-adherent bacteria were removed by pipetting and washed with 3 mL of sterile saline. Aliquots of 2 mL of sterile TSB-YE were inoculated into each well. Coupons were incubated over 7 days at 25°C and washed at 2-days intervals, and wells were inoculated with 2 mL of fresh TSB-YE.

#### *Scanning electron microscopic (SEM) analysis of biofilm on stainless steel*

After 7 days of incubation the coupons were removed from the well and washed by mild pipetting with 3 mL of sterile saline to remove the medium and non-adhered cells. The coupons were then fixed overnight at 5°C in 4% formaldehyde, double-washed in sterile distilled water and were dehydrated in serial dilutions of 30, 50, 60, 70, 90% ethanol for 5 min each, followed by three 10-min rinses in 100% ethanol. Air-dried preparations were immediately sputter-coated with gold (Sputer Coater SCD 005, BALTEC SCAN, WD=50 mm, 90 s, 30 mA) and examined using scanning electron microscope JMS SEM 6460 LV, (acceleration voltage 25 KV, at WD 20 to 8 mm).

## RESULTS AND DISCUSSION

The results of scanning electron microscopy are displayed in micrographs, and for most strains the magnification was as low as 2000 or 5000. Lower magnification enables an insight in the coating rate of the coupon surface, i.e. ability of the examined strain to colonize the surface of stainless steel. For particular bacterial strains the details are displayed at magnifications 20 000 – 45 000.

Seven of 14 tested strains of *L. monocytogenes* did not form the biofilm in given conditions, but adhered to the surface as isolated cells distributed over the stainless steel surface (Fig.1.A-H). Similar results were reported by Kalmokoff *et al.* (2001). Using the scanning electron microscopy these authors established that 35 of the total of 36 investigated strains of *L. monocytogenes* did not form the biofilm and only individual bacterial cells adsorbed to the stainless steel surface.

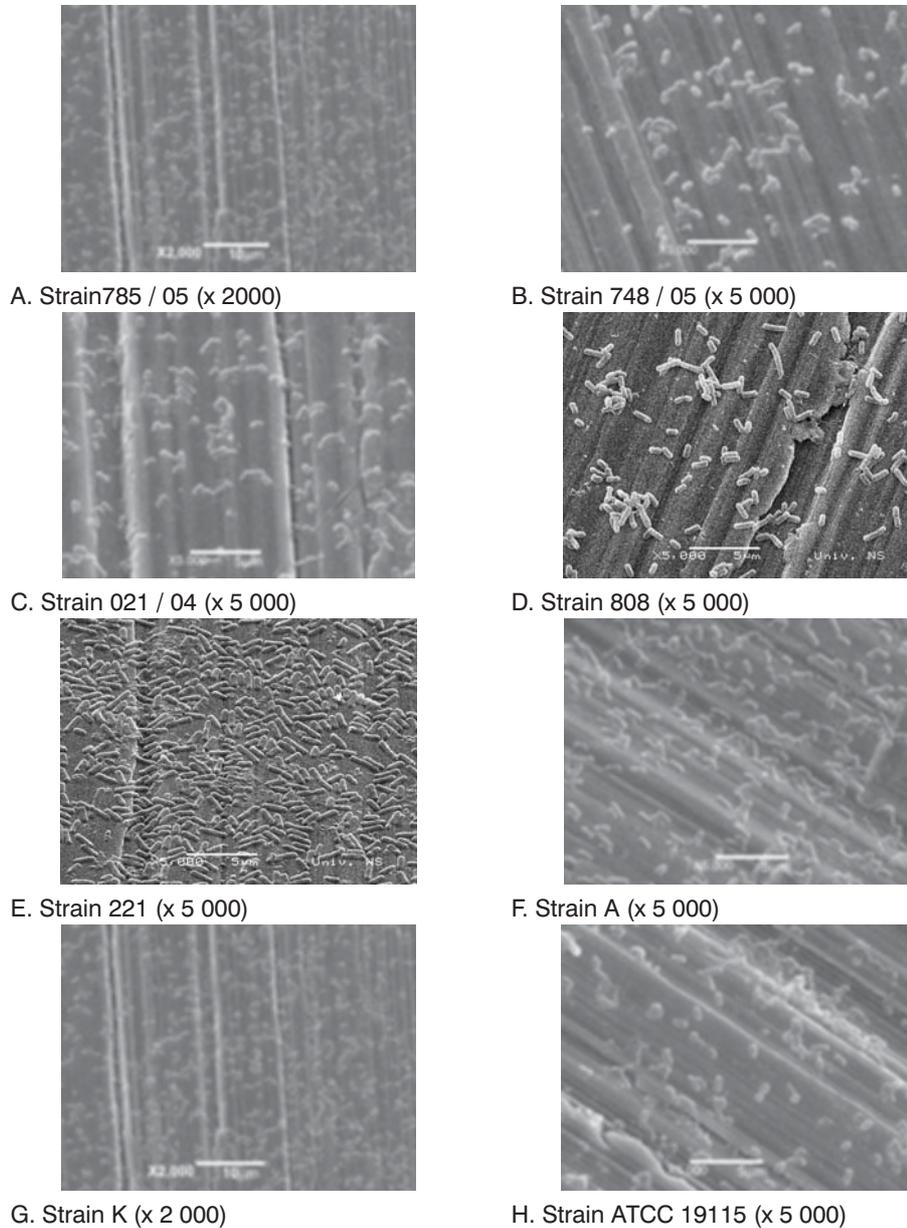
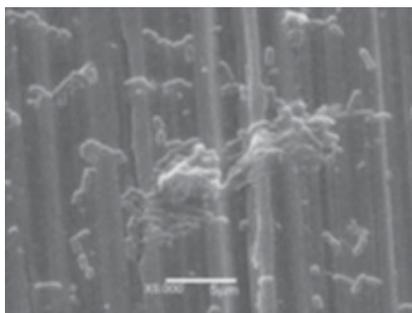
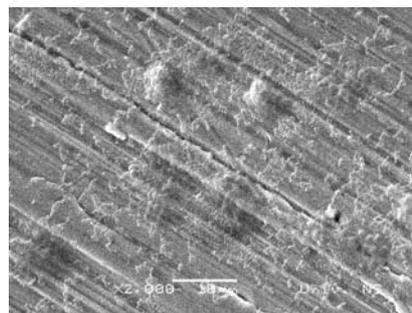


Figure 1. SEM images – strains of *Listeria monocytogenes* that did not form biofilms on stainless steel for 7 days of incubation in TSB at 25°C. Bars 5 µm (magnification 5 000); bars 10 µm (magnification 2 000)

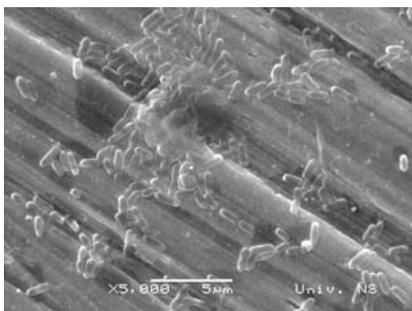
Only one strain (serotype 4b) originating from foodstuff exhibited the ability of forming microcolonies and larger cells aggregates, i.e. a "rudimentary biofilm". In this experiment the formation of discrete cell aggregates was observed along the cracks in the stainless steel in the control strain ATCC 19115 (Fig.1.H), and to somewhat greater extent in the strain 1915/04 (Fig.2.A). Blackman and Frank (1996) also documented the formation of microcolonies by some strains of *L. monocytogenes* following long-term incubation with multiple transfers of the test surfaces into the fresh nutritive medium.



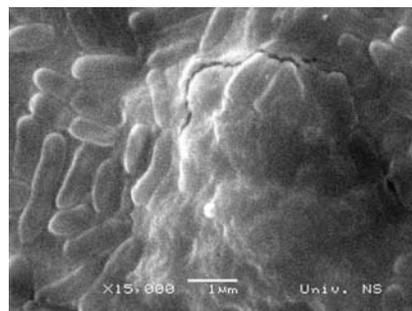
A. Strain 1915 / 04 (x 5 000)



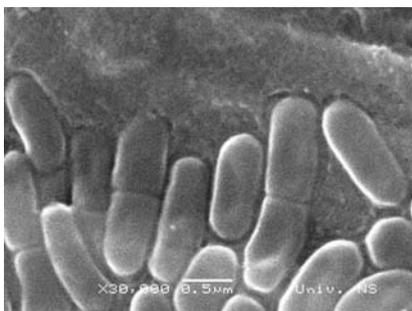
B. Strain 593 / 05 (x 2 000)



C. Strain 593 / 05 (x 5 000)



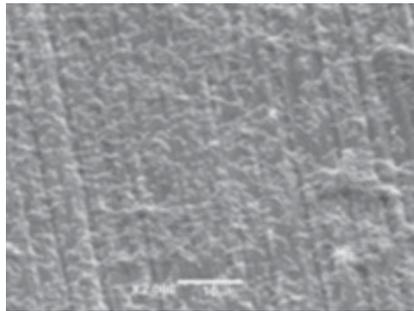
D. Strain 593 / 05 (x 15 000)



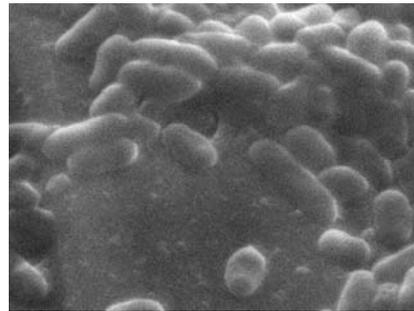
E. Strain 593 / 05 (x 30 000)

Figure 2. SEM images – three-dimensional cell aggregates of *Listeria monocytogenes* strains 1915/04 i 593/05 on stainless steel after 7 days of incubation in TSB at 25°C

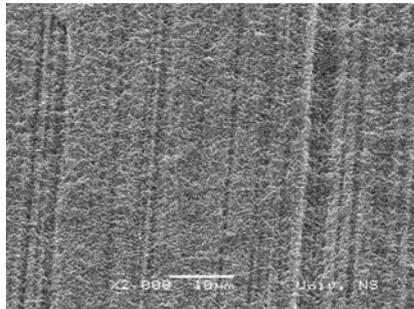
The strain *L. monocytogenes* 593/05 did not form a uniform distribution of cells across the entire steel surface, with uncovered regions clearly visible at lower magnification. Concurrently, the colonized areas reveal a "hilly landscape" characterized by large three-dimensional cell aggregates (Fig.2.B,C). The colonization rate in strains *L. monocytogenes* F and 154 was higher than that of the strain 593/05, revealing moderately elevated three-dimensional cell aggregates on the stainless steel surface (Fig.3. A-C).



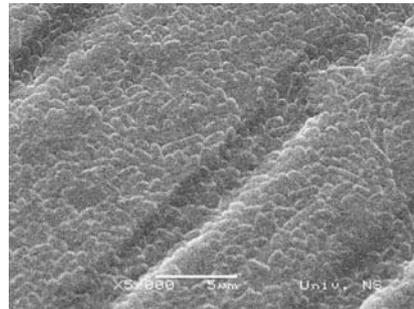
A. Strain F (x 2 000)



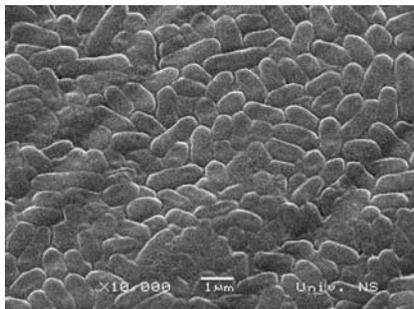
B. Strain F (x 20 000)



C. Strain 154 (x 2 000)



D. Strain 16 (x 5 000)

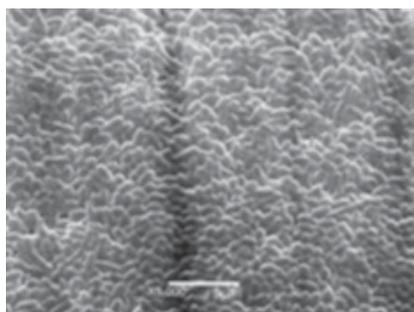


E. Strain 16 (x 10 000)

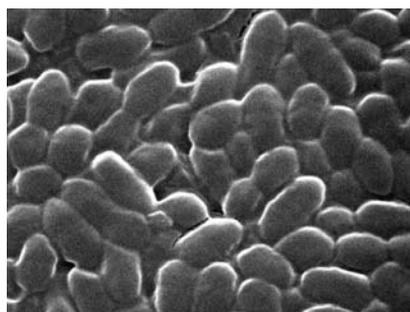
Figure 3. SEM images – biofilms of *Listeria monocytogenes* strains F, 154 i 16 on stainless steel after 7 days of incubation in TSB at 25°C

Strain 16 formed a uniform monolayer over the entire stainless steel surface without tending to form cell aggregates (Fig.3.D and E). The best ability of colonizing stainless steel surface and formation of characteristic cell aggregates was observed in strain C, which completely covered the stainless steel surface, thus grooves on the surface that are characteristic for the finishing of this material are visible only as somewhat darker contours (Fig.4.A-C). Larger magnifications revealed two- or multiple- layer cell arrangement of the structures.

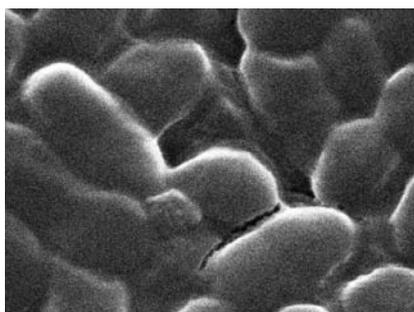
The obtained results strongly imply that there are substantial differences among *Listeria monocytogenes* strains with respect to their ability of colonizing the stainless steel surface and biofilm formation. In spite of quite a small number of isolates used in this testing this ability can not be attributed to the serological type of the organism. Chae and Schraft (2000) also could not establish the correlation between the serotype and origin of *L. monocytogenes* strains and their ability of adhering and forming biofilm on the stainless steel surface. Kalmokoff *et al.* (2001) established significant differences in the number of adhered cells among different strains of *L. monocytogenes*. However, the correlation between serotype or strain origin could not be confirmed.



A. Strain C (magnification 5 000)



B. Strain C (magnification 20 000)



C. Strain C (magnification 45 000)

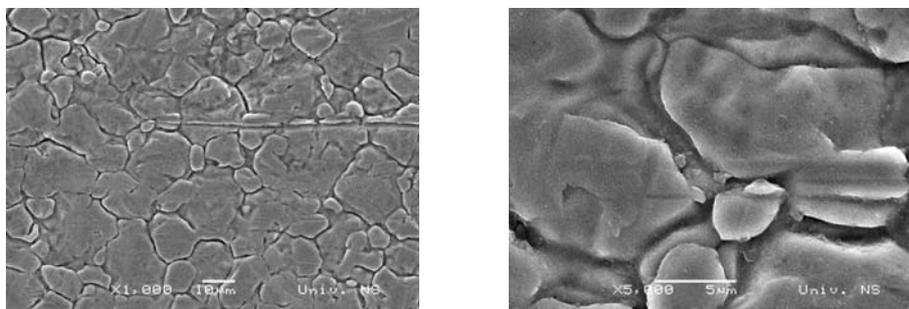
Figure 4. SEM images – biofilm of *Listeria monocytogenes* strain C on stainless steel after 7 days of incubation in TSB at 25°C

The ability of biofilm formation in different *L. monocytogenes* strains is related to the individual features of the strain, its persistence (Norwood and Gilmour, 1999; Borucki *et al.*, 2003; Lunden *et al.*, 2000) and phylogenetic origin (Đorđević *et al.*, 2002). Furthermore, experimental conditions such as incubation period and temperature, composition of nutritive medium and surface (substrate) characteristics, are of paramount importance. The control strain Scott A used in the research of Kalmokoff *et al.* (2001) did not form biofilm on the stainless steel surface at room temperature for 72h in the brain heart infusion broth. Experiment of Marsh *et al.* (2003) demonstrated that, in experimental conditions at 35°C in TSB, the same strain formed a biofilm of "honeycomb structure" with a clearly pronounced three-dimensional structure. The extracellular polymeric substance (EPS) observed in all biofilms of *Listeria monocytogenes* strains in this study were thread-like, binding one cell to another and to the surface. Chae and Schraft (2000) attribute the two-layer structure of the biofilm formed by two *L. monocytogenes* strains on glass surface to rather static conditions used in this experiment. According to the authors, the bacteria attached to the surface produce daughter cells that have only a loose association with EPS, because there are no constant external forces, such as the shear forces present in a continuous flow system, to keep cells attached more strongly and to promote the formation of common "mushroom-shaped" structures of microbial biofilms.

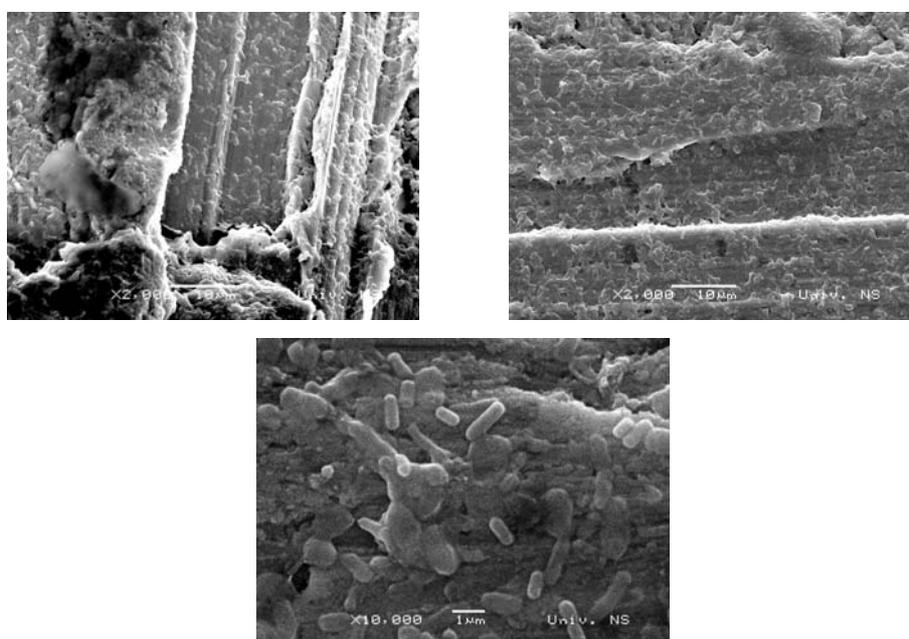
The described biofilm structures of *Listeria monocytogenes* distinctively differ from the commonly described "mushroom" biofilm structure, where bacterial cells (such as *Staphylococcus spp.*) are completely embedded in a thick, sticky extracellular substance. Marsh *et al.* (2003) report that *Listeria monocytogenes* biofilm architecture is probably more similar to a porous, sponge-like biofilm structure characteristic for *Streptococcus mutans*. Different types of EPS synthesized by bacteria or different signals involved in biofilm development could all contribute to these structural differences.

Chmielewski and Frank (2003) report that the biofilm appearance may vary from the single layer to the three-dimensional structure or take the form of aggregates such as flocks or granules.

In order to estimate the „span“ of the ability of *L. monocytogenes* to form a biofilm, in this experiment we used an isolate of *Pseudomonas aeruginosa* isolated from the milk of a cow with clinical mastitis. *Pseudomonas* species are well established good producers of biofilm and EPS. The preparation was made using a bacterial suspension of *P. aeruginosa* of the same density as for the tested strains of *L. monocytogenes* ( $10^7$  cfu/mL), and the biofilm was prepared under the same conditions, with an incubation period of 7 days at 25°C. *Pseudomonas aeruginosa* formed a dense three-dimensional biofilm with large amounts of ESP that completely covered the bacterial cells, thus they could not be detected by examining the upper coupon surface (Fig. 5.A). At the surface of the withered (condensed) matrix the cracks and pores were clearly obvious, which correspond to the appearance of water channels among bacterial microcolonies that transport nutrients, water and oxygen to the cells and excrete metabolic products. The cells of *P. aeruginosa* were detected only at the side parts of the coupons, where they were not covered by a biofilm matrix (Fig. 5.B). In comparison with the



A. Appearance of the upper coupon surface with *Pseudomonas aeruginosa* biofilm (magnification 1000 and 5000)



B. Appearance of the side coupon surface with *Pseudomonas aeruginosa* biofilm (magnification 2 000 and 10 000)

Figure 5. SEM images – biofilm of strains *Pseudomonas aeruginosa* on stainless steel after 7 days of incubation in TSB at 25°C

biofilm formed by *P. aeruginosa*, tested strains of *L. monocytogenes* exhibited a weak or moderate ability of biofilm formation, and particularly a weak ability of producing the extracellular polymeric substance. In strain 593/05 cells covered with withered mass that may correspond to the biofilm matrix (Fig. 2.D) are visible at a larger magnification. Prints of bacterial cells that were apparently embedded

in this substance are clearly noticeable at a magnification of 30 000 (Fig. 2.E). Such an appearance of the extracellular substance differs from the filamentous structures described by Kalmokoff *et al.* (2001), Borucki *et al.* (2003) and Marsh *et al.* (2003).

Considering the soft, highly hydrated structure of the biofilm the preparation of the substance for SEM requires a gradual dehydration procedure, because water is not compatible with the vacuum used for the electron ray. Dehydration results in substantial deformation of the biofilm matrix; however, excellent features of the electron microscopy resolution still make this technique highly applicable and important in biofilm research.

Further research is necessary to understand differences among strains of *L. monocytogenes* and their ability of biofilm formation. Knowledge obtained in this area will represent the first step in prevention of biofilm formation and elimination of persistent bacterial strains in the food processing environments (Harvey *et al.* 2007).

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Address for correspondence:  
Dr Dubravka Milanov  
Scientific Veterinary Institute "Novi Sad"  
Rumenački put 20, Novi Sad  
Serbia  
e-mail: dubravka@niv.ns.ac.yu

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## **SKENING ELEKTRONSKA MIKROSKOPIJA BIOFILMOVA SOJEVA *LISTERIA MONOCYTOGENES* NA NERĐAJUĆEM ČELIKU**

MILANOV DUBRAVKA, AŠANIN RUŽICA, VIDIĆ BRANKA, KRNJAIĆ D,  
PETROVIĆ JELENA i SAVIĆ SARA

### **SADRŽAJ**

*Listeria monocytogenes* je uzročnik brojnih epidemija i sporadičnih slučajeva oboljenja ljudi, a hrana predstavlja glavni put prenošenja infekcije. Sirovine biljnog i animalnog porekla su jedan od mogućih izvora kontaminacije namirnicama ovom bakterijom, što se posebno odnosi na prehrambene proizvode koji prolaze minimalni proces obrade. Međutim, poslednjih godina sve više se ističe značaj postprocesne kontaminacije, nastale kao rezultat kontakta prehrambenih proizvoda sa kontaminiranim površinama u pogonima prehrambene industrije, na kojima ova bakterija može u formi biofilma opstati mesecima, pa čak i godinama.

Uprkos skoro opšte prihvaćenom stavu da *Listeria monocytogenes* formira biofilm, malo je mikroskopskih dokaza koji to potvrđuju. U ovom radu ispitana je sposobnost formiranja biofilma kod 14 sojeva *Listeria monocytogenes* različitog porekla. Biofilmovi su formirani na površini nerđajućeg čelika za sedam dana

inkubacije u tripton soja bujonu sa dodatkom 0.6% kvašćevog ekstrakta na temperaturi od 25°C. Formirane strukture pregledane su skening elektronskom mikroskopijom.

Rezultati pregleda potvrđuju da među testiranim sojevima *Listeria monocytogenes* postoje značajne razlike u sposobnosti da formiraju biofilm pod korišćenim uslovima ispitivanja. Sedam od 14 testiranih sojeva *Listeria monocytogenes* nije formiralo biofilm, već su se njihove ćelije kao pojedinačne rasporedile po površini nerđajućeg čelika. Sojevi koji su procenjeni kao "biofilm producers" formirali su strukture različitog izgleda, od uniformnog, konfluentnog monosloja bakterijskih ćelija do pojedinačnih krupnih, trodimenzionalnih ćelijskih agregata. Svi ispitani sojevi su slabo produkovali ekstracelularnu polimeričnu supstanciju.