Acta Veterinaria (Beograd), Vol. 57, No. 5-6, 429-440, 2007.

DOI: 10.2298/AVB0706429M UDK619:616.9-092.9

INVESTIGATION OF BIOFILM FORMATION *IN VITRO* ABILITY OF *LISTERIA MONOCYTOGENES* STRAINS ISOLATED FROM ANIMALS

MILANOV DUBRAVKA*, AŠANIN RUŽICA**, MIŠIĆ D** VIDIĆ BRANKA* and RATAJAC R*

**Scientific Veterinary Institute "Novi Sad", Novi Sad;*
***Faculty of Veterinary Medicine, Belgrade*

(Received 2. March 2007)

Listeria monocytogenes is the causative agent of listeriosis in humans and animals and an important food-born pathogen. Control of its presence in food processing plants, particularly on sites where food contamination is expected, is of paramount importance with respect to food safety and protection of human health. Numerous studies demonstrated that this organism can be isolated during several months, even several years, from diverse sites in food processing plants, which is due to its ability to adsorb onto inert surfaces and form a biofilm, alone or in coexistence with other bacterial species.

In this study we investigated the ability of 16 animal isolates of L. monocytogenes to form a biofilm on polystyrene microtiter plates. The investigation was performed at three different temperatures 4°C, 25 °C and 37°C that are commonly suitable for growth of Listeria monocytogenes. The research was carried out using three different nutritive media: trypthon-soy broth with yeast extract (TSB-YE), brain-heart infusion (BHI) and 1/20 diluted trypthon-soy broth with yeast extract (1/20 TSB-YE). In order to investigate the biofilm formation in vitro an inoculum was prepared from 24-hours-old cultures of isolated strains of L.monocytogenes. The density of the inoculum was 2-10x10^7 cfu/mL (OD_{600}=0.093 ± 0.009 in TSB-YE). The microtiter plates were incubated at cited temperatures during 48h. Colonization rate of L.monocytogenes strains on polystyrene surface and biofilm formation were monitored using crystal violet stain added to the microtiter plates, as well as using light microscopy. The tested strains demonstrated a diverse ability of biofilm formation, depending on the incubation temperature and nutritive medium. In this paper we presented the results obtained for four strains of Listeria monocytogenes isolated from brain samples of sheep, designated as 785/05, 593/05, 748/05, 1915/04 and one strain isolated from aborted calve's fetus, designated as 021/04. The referent strain of L.monocytogenes, 1071 (4b), was used as a control. Strains of L. monocytogenes cultured in nutrient-rich media (e.g. TSB-YE) and at higher temperatures (37°C) exhibited a higher ability of biofilm formation.

However, non of the studied strains could be classified as a good biofilm producer, disregard of the incubation temperature and medium used. The highest OD values were obtained at the incubation temperature of 37°C in TSB-YE (OD_{595}=0.346 ± 0.055), when three strains were quantified as moderate and two as weak biofilm producers. The decrease of incubation temperature resulted in decreased OD values, thus four strains were classified as weak biofilm producers at 25°C (OD_{595}=0.289 ± 0.083), and none of the investigated strains was assessed as biofilm producers at 4°C (OD_{595}=0.124 ± 0.011)

Light-microscopy examination, confirmed by quantitative values obtained by the crystal violet microtiter test, proved to be a simple and rapid screening method for the quantification of the ability of L.monocytogenes to form biofilms in varying test conditions.

Key words: Listeria monocytogenes, biofilm, microtiter test, crystal violet stain

INTRODUCTION

Until the 1970' listeriosis was mainly reported as a disease of farm animals fed on silage. The source of human infection was not known until the 1980', when three subsequent case-control studies after large listeriosis outbreaks in North America clearly indicated the food borne nature of this illness. *L. monocytogenes* is ubiquitously distributed in the environment. The organism is frequently isolated from a diversity of environmental sources, including soil, water, plants, a large variety of foods, as well as feces of humans and animals. The prevalence of listeriosis is rather law in spite of high exposure rate, which may be due to differences in *L. monocytogenes* virulence properties, as well as the existence of vulnerable groups within human populations (Notermans *et al*., 1998).

Although *L. monocytogenes* is clearly a pathogenic organism, not all infections result in serious illness. Capacity of particular strains of *L.monocytogenes* to provoke the disease is determined by several factors, including its ability of persisting in the environment where possible food contamination is expected. Several studies indicated that raw materials were not a major source of contamination, but that contamination occurred during processing and the food-processing equipment can act as a reservoir of *L. monocytogenes* (Vogel *et al*., 2001; Møreto and Langsrud 2004).

The abundant data from the available literature clearly indicate that *L. monocytogenes* can adsorb to various surfaces and materials used in food processing industry, such as stainless steel, glass, plastic, rubber, polycarbonate surfaces (Mafu *et al*., 1990; Jeong and Frank, 1994; Chmielewski and Frank 2003). Thus, the organism adsorbed at the surface is capable to form a biofilm (Rönner and Wong 1993; Blackman and Frank, 1996; Chmielewski and Frank, 2003) or to grow and persist in biofilms formed by other bacterial species (Hood and Zottola, 1997; Carpentier and Chassaing, 2004). It was experimentally demonstrated that

the ability of *L. monocytogenes* to form biofilms is associated with its serotype and persistence (Lunden *et al.*, 2000; Chae and Schraft, 2000; Harvey *et al*., 2007), phylogenetic division (Đorđević *et al.,* 2002; Borucki *et al*., 2003), and coexistence of other microorganisms (Hood and Zottola*,* 1997; Leriche and Carpentier, 2000; Norwood and Gilmour, 2001; Carpentier and Chassaing, 2004).

A biofilm is defined as a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extra cellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription (Donlan and Costerton, 2002). Bacteria in a biofilm demonstrate an increased resistance to antimicrobial agents, including antibiotics and disinfectants, presenting a persistent source of food contamination with food spoilage bacteria, as well as with pathogenic organisms.

In order to enable an easier study of bacterial attachment and colonization, a variety of direct (various microscopy techniques) and indirect methods have been developed. Indirect methods are based on detaching the organisms from the surface and determining their count (roll techniques and sonication, standard plate counts) or on mediated estimation of the number of attached organisms *in situ* by measuring some of their attributes (radiolabeled bacteria, enzyme-linked immunosorbent assay, biologic assays, stained bacterial films and microtiter plate procedures) (Đorđević *et al*., 2002). Crystal violet test in microplates is described as a simple and applicable method for determining the ability of different bacterial species to form biofilms.

The objective of the present study was to apply the microtiter plate assay to determine the abilities of some clinical isolates of *L. monocytogenes* strains to form biofilms in different nutritive media (*Tryptone soy broth* with yeast extract 6 g/L (TSB-YE), 1/20 diluted TSB-YE and *brain heart infusion broth* – BHI) at different incubation temperatures (4°C, 25 ^{o}C and 37°C). Concomitantly with the microtiter plate assay, adhesion of investigated strains of *L. monocytogenes* to polystyrene was assessed by the method of light microscopy.

MATERIAL AND METHODS

Bacterial strains

The investigation was performed using five clinical isolates of *L. monocytogenes* and a control strain of *L. monocytogenes* 1071. Four strains (785/05, 593/05, 748/05 and 1915/04) were isolated from the brain tissue and medulla oblongata of sheep died with signs of CNS disease, and one strain (021/04) was isolated from aborted fetuses of cows. Identification of *L. monocytogenes* was performed using the microscopy technique (Gram staining), catalase and oxidase tests and a double CAMP test (*Staphylococcus aureus* and *Rhodococcus equi,* control strains *Streptococcus agalactiae* and *Listeria ivanovii*). Biochemical properties of the isolates were examined using the MICROBACT^{TM} Listeria Identification System (Oxoid, Basingstoke, UK).

Culture media

Culture media used in this study were as following: *Tryptone soy broth* (TSB) with 0.6% yeast extract (Yeast extract, Biokar Diagnostics), *brain heart infusion broth* (BHI, Biokar Diagnostics) and TSB-YE diluted 20-fold in saline.

Preparation of Listeria monocytogenes cultures for CV microtiter plate assay

In order to obtain single colonies all strains of *L.monocytogenes* were cultured on blood agar with 5% sheep blood that was incubated for 24 h at 37°C. Three to four individual 24-hour-old colonies of each testing strain were picked, inoculated in 3 mL of TSB-YE and incubated for 24h at 37°C. In order to prepare the inoculum for the crystal violet test in microplates, the suspensions were homogenized in a vortex mixer and diluted to 1:40 in the TSB-YE, BHI and in 1:20 saline dilution of TSB-YE. The density of the inoculum was 2-10x10^7 cfu/mL (OD_{600}=0.093 (±0.009) in TSB-YE).

Microtiter plate assay for biofilm formation:

Test at 37°C

Aliquots of 100 μL of a single strain suspension were dispensed into each of eight wells of a sterile polystyrene plate for cell cultures (NUNC, Roskilde, Denmark). Each plate also included a set of eight wells filled only with the tested medium as a negative control.

Plates were sealed and incubated at 37°C for 48h without agitation. After 48h incubation period, the medium was removed using a pipette, and the microtiter plate wells were washed three times with sterile distilled water (200 μL-aliquots) to remove loosely associated bacteria. Plates were then inverted and blotted on paper towels and allowed to dry for 30 min. Each well was stained with 100 μL 0.2% crystal violet (Sigma) solution in water for 45 min at room temperature.

The unbound stain was removed by multiple washing with sterile water until no more visible traces of dye were present in the solution. To dissolve the stain bound to the bacteria (biofilm) aliquots of 100 μL of 95% ethanol were added to each well and incubated at 4°C for 30 minutes. Cell turbidity was monitored using a spectrophotometer (Labsystems Multiskan® MCC/340) at an optical density at 595 nm (OD_{595}).

Test at 25°C and 4°C

The test was performed according to the same procedure as described for 37°C, but suspensions of tested strains were incubated at 25°C. The suspensions were then inoculated on two microplates and incubated at 25°C and 4°C.

Calculations:

The microplate biofilm assay was repeated on three separate days for all *L. monocytogenes* strains, and the averages and standard deviations for each strain were calculated.

Calculations: Based on OD values the investigated strains were classified into categories as following: strains that do not produce biofilm, weak, moderate and strong biofilm producers (Stepanović *et al*., 2004). Cut-off O.D. (O.D.c) was defined as three standard deviations above the median value of O.D. negative control. The strains were classified as follows:

1. strains that do not produce a biofilm: O.D. ≤ O.D.c;
2. weak biofilm producers: O.D.c < O.D. < (2 x O.D. c);
3. moderate biofilm producers (2 x O.D.c) < O.D. ≤ (4 x O.D.c);
4. strong biofilm producers (4 x O.D.c) < O.D.

Microscopic biofilm formation assay:

Aliquots of 0.5 mL of bacterial suspensions (prepared for the microtiter plate test) were inoculated in the wells of polystyrene plate Linbro® (Flow Laboratories, Virginia, USA). The plates contain 4 wells, and are 42.4 mL in volume and 28.2 cm^2 in size. Aliquots of 5 mL of the tested medium were added in the wells, and plates were then incubated for 48h at 25°C and 37°C. After the incubation period the liquid was removed, and plates were washed three times with 10 mL sterile saline, inverted and allowed to dry. Bacteria were stained with 0.2% crystal violet for 45 minutes at room temperature. The unbound stain was removed by washing. The plates were then examined employing light microscopy technique, using *Olympus* microscope (Tokyo) (20x).

RESULTS AND DISCUSION

The crystal violet microtiter test was described in the literature as a simple and rapid method to quantify biofilm formation of different bacterial strains (Đorđević *et al*., 2002; Borucki *et al*., 2003; Stepanović *et al.,* 2004; Harvey *et al*., 2007). In this research, we applied procedures based on methods described by Đorđević *et al*. (2002) and Borucki *et al*. (2003). Contrary to the cited procedures, we used equal volumes of culture suspensions of the tested organisms, stain solution and alcohol for decolorization. Furthermore, the test was carried out on polystyrene plates since extinction quantification on PVC surface requires transferring the liberated dye (following cell lysis) to the new polystyrene plates (Broschat *et al.,* 2005).

The median extinction values at OD_{595} for three repeated tests ranged from 0.109 to 0.42 and are displayed in Tables 1, 2 and 3.

Neither of the investigated strains was assessed as strong biofilm producer, disregarding the incubation temperature and medium used. The highest OD values were obtained at incubation temperature 37°C in TSB-YE (OD_{595}=0.346 ± 0.055). Under the same conditions three strains were quantified as moderate and two as weak biofilm producers. Decrease of incubation temperature resulted in decreased OD values in the same medium, thus four strains were classified as weak biofilm producers at 25°C (OD_{595}=0.289 ± 0.083), and none of the investigated strains was assessed as biofilm producers at 4°C (OD_{595}=0.124 ± 0.011).

Table 1. Median extinctions values (OD_{595}) measured in three repeated crystal-violet tests in microtiter plates - incubation temperature 37°C

Strain	OD_{595} in TSB at 37°C	OD_{595} in BHI at 37°C	OD_{595} in 1:20 TSB at 37°C
785/05	0.403 ± 0.116)**	0.345 ± 0.072)**	0.184 ± 0.029) *
593/05	0.358 ± 0.059)**	0.189 ± 0.024)*	0.188 ± 0.029) *
748/05	0.275 ± 0.05)*	0.336 ± 0.072)**	0.156 ± 0.041) *
021/04	0.390 ± 0.086)**	0.363 ± 0.062)**	0.233 ± 0.078) *
1915/04	0.306 ± 0.059)*	0.218 ± 0.027)*	0.221 ± 0.035) *
Control	0.349 ± 0.068)**	0.249 ± 0.045)*	0.202 ± 0.05) *
N.K.	0.103 ± 0.018)	0.099 ± 0.019)	0.104 ± 0.02)
ODc	0.157	0.156	0.164

Table 2. Median extinctions values (OD_{595}) measured in three repeated crystal-violet tests in microtiter plates - incubation temperature 25°C

Strain	OD_{595} in TSB at 25°C	OD_{595} in BHI at 25°C	OD_{595} in 1:20 TSB at 25°C
785/05	0.397 ± 0.068)**	0.420 ± 0.106)**	0.193 ± 0.029)*
593/05	0.178 ± 0.024)*	0.217 ± 0.046)*	0.188 ± 0.054)*
748/05	0.332 ± 0.039)*	0.239 ± 0.039)*	0.168 ± 0.036)*
021/04	0.292 ± 0.051)*	0.201 ± 0.032)*	0.176 ± 0.029)*
1915/04	0.246 ± 0.048)*	0.189 ± 0.042)*	0.191 ± 0.029)*
Control	0.354 ± 0.078)**	0.335 ± 0.087)*	0.189 ± 0.067)*
N.K.	0.114 ± 0.021)	0.118 ± 0.018)	0.102 ± 0.015)
ODc	0.177	0.172	0.147

Table 3. Median extinctions values (OD_{595}) measured in three repeated crystal-violet tests in microtiter plates - incubation temperature 4°C

Strain	OD_{595} in TSB at 4°C	OD_{595} in BHI at 4°C	OD_{595} in 1:20 TSB at 4°C
785/05	0.111 ± 0.011)°	0.115 ± 0.007)°	0.119 ± 0.008)°
593/05	0.118 ± 0.011)°	0.109 ± 0.008)°	0.121 ± 0.009)°
748/05	0.126 ± 0.022)°	0.123 ± 0.033)°	0.162 ± 0.066)*
021/04	0.124 ± 0.03)°	0.121 ± 0.006)°	0.179 ± 0.045)*
1915/04	0.140 ± 0.019)°	0.149 ± 0.047)°	0.159 ± 0.016)*
Control	0.126 ± 0.016)°	0.116 ± 0.017)°	0.148 ± 0.043)*
N.K.	0.102 ± 0.019)	0.102 ± 0.015)	0.099 ± 0.009)
ODc	0.159	0.147	0.126

° – not a biofilm producer; * – weak biofilm producer; ** – moderate biofilm producer

Incubation temperature may influence the cell wall composition and thereby modify the surface electrical properties, hydrophobicity and electron donor or electron acceptor character of the bacterial cell (Chavant *et al*., 2002). At low temperatures the cell surface of *L. monocytogenes* is strongly hydrophilic at either growth phase, which suggests some modifications in cell wall composition (Chavant *et al*., 2002). Increased hydrophilicity of cells at low temperatures makes colonization of the hydrophobic substratum (polystyrene) very difficult or even impossible, which could explain extremely low OD values at this temperature in all used media.

CV microtiter assay demonstrated a strong influence of the composition of nutritive medium on biofilm production by *L. monocytogenes*. The obtained median extinction values were higher in TSB-YE in comparison with the BHI and 1/20 diluted TSB-YE, both at 37°C and 25°C. The lowest extinction values were obtained at all temperatures in diluted TSB-YE. Somewhat higher values were recorded in BHI at 37°C ($OD_{595}=0.29\pm0.08$) in comparison with the 25°C ($OD_{595}=0.253\pm0.095$); however, these differences are not significant.

Our results demonstrated that *L. monocytogenes* is a better biofilm producer in reach nutritive media, which corresponds with the results of Hood and Zottola (1997), Stepanović *et al*. (2004) and Harvey *et al*. (2007). However, the trial conducted by Stepanović *et al*. (2004) revealed higher extinction values by culturing in BHI than in TSB. Diluted TSB, which is usually applied to simulate conditions in food processing industry (Stepanović *et al*., 2004), positively influenced the biofilm production by tested *Salmonella* strains, while strains of *L. monocytogenes* revealed lowest values in the same medium, which corresponds to our results. Rönner and Wong (1993) and Blackman and Frank (1996) report that nutrient reduction results in reduced attachment of *L. monocytogenes*. Decrease in concentration of nutritive compounds reduces growth of *L. monocytogenes*; however, the complex mechanism of biofilm growth regulated by diverse factors is still poorly understood (Harvey *et al*., 2007).

The value of crystal violet microtiter assay is most likely to be pronounced in parallel examination of several different strains of the same species or different bacterial species. However, crystal violet assay quantifies only the amount of dye bound to the bacteria, while the extra exopolysaccharides characteristic for the biofilm are not detected (Broschat *et al.* 2005). Parallel with the crystal violet assay, Borucki *et al*. (2003) stained some of the investigated strains with ruthenium-red, the dye that specifically binds to carbohydrates. The authors established that *L. monocytogenes* produces some types of extracellular carbohydrates coexistent with the biofilm matrix, yet the microtiter plate assay proved to be not sensitive enough because of the small number of adhered cells.

Fig. 1. displays the microscopic appearance (light microscopy, 20x) of the biofilm of three tested strains (593/05, 748/05 and 021/04) developed on polystyrene plates after 48h at 37 and 25°C in all nutritive media. At incubation temperature of 37°C in TSB-YE and BHI the strains developed a network structure similar to honey-comb, and such appearance of the biofilm obtained from light microscopy was described in the literature for diverse strains of *L. monocytogenes* (Đorđević *et al*., 2002; Marsh *et al*., 2003; Wang, 2004). Between

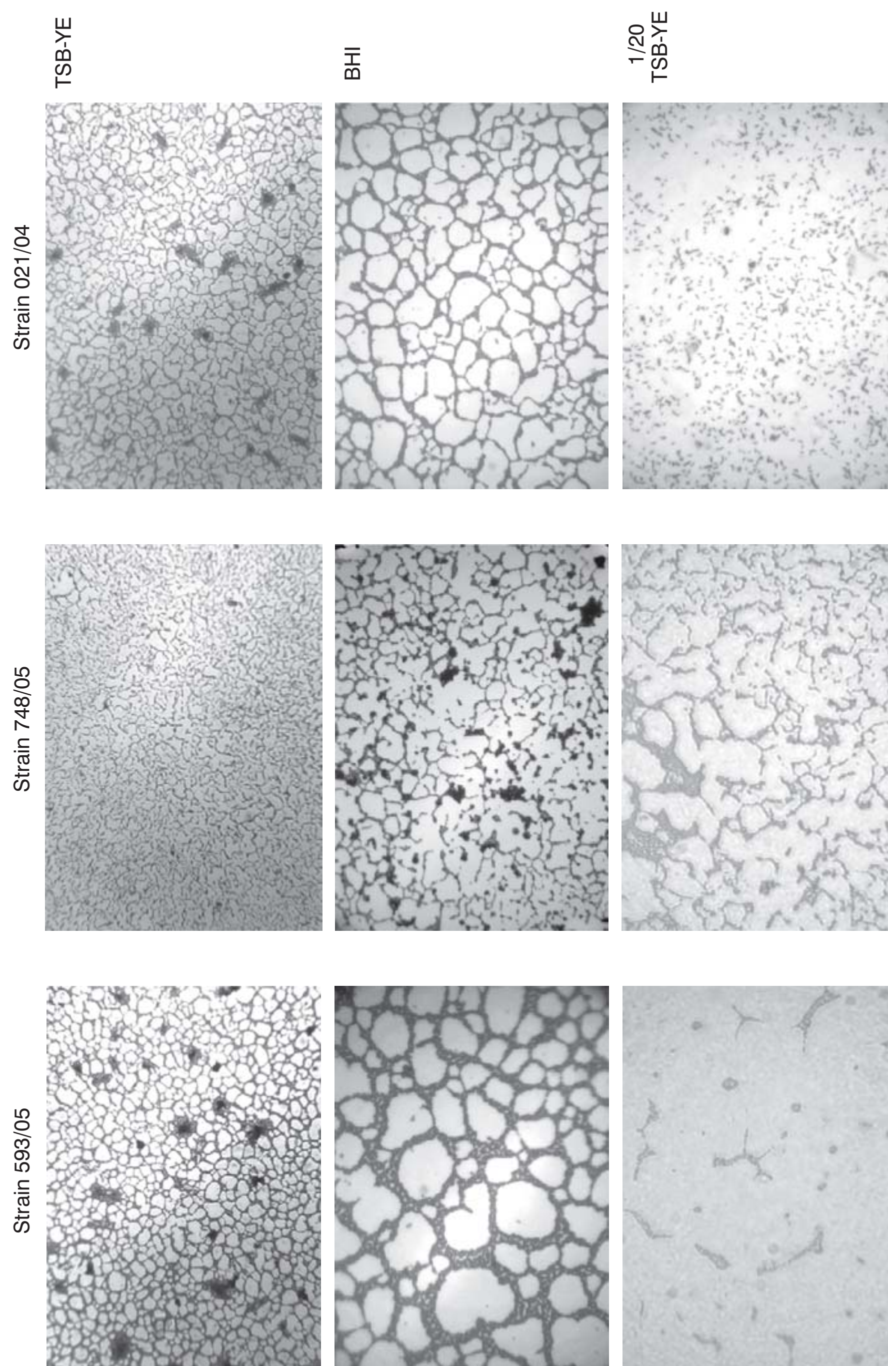

Figure 1. Light microscopy (20x) of *L. monocytogenes* strains 593/05, 748/05 and 021/04 the biofilm on polystyrene surface, at 37ºC, incubation period 48 h, in TSB, BHI and 1/20 TSB

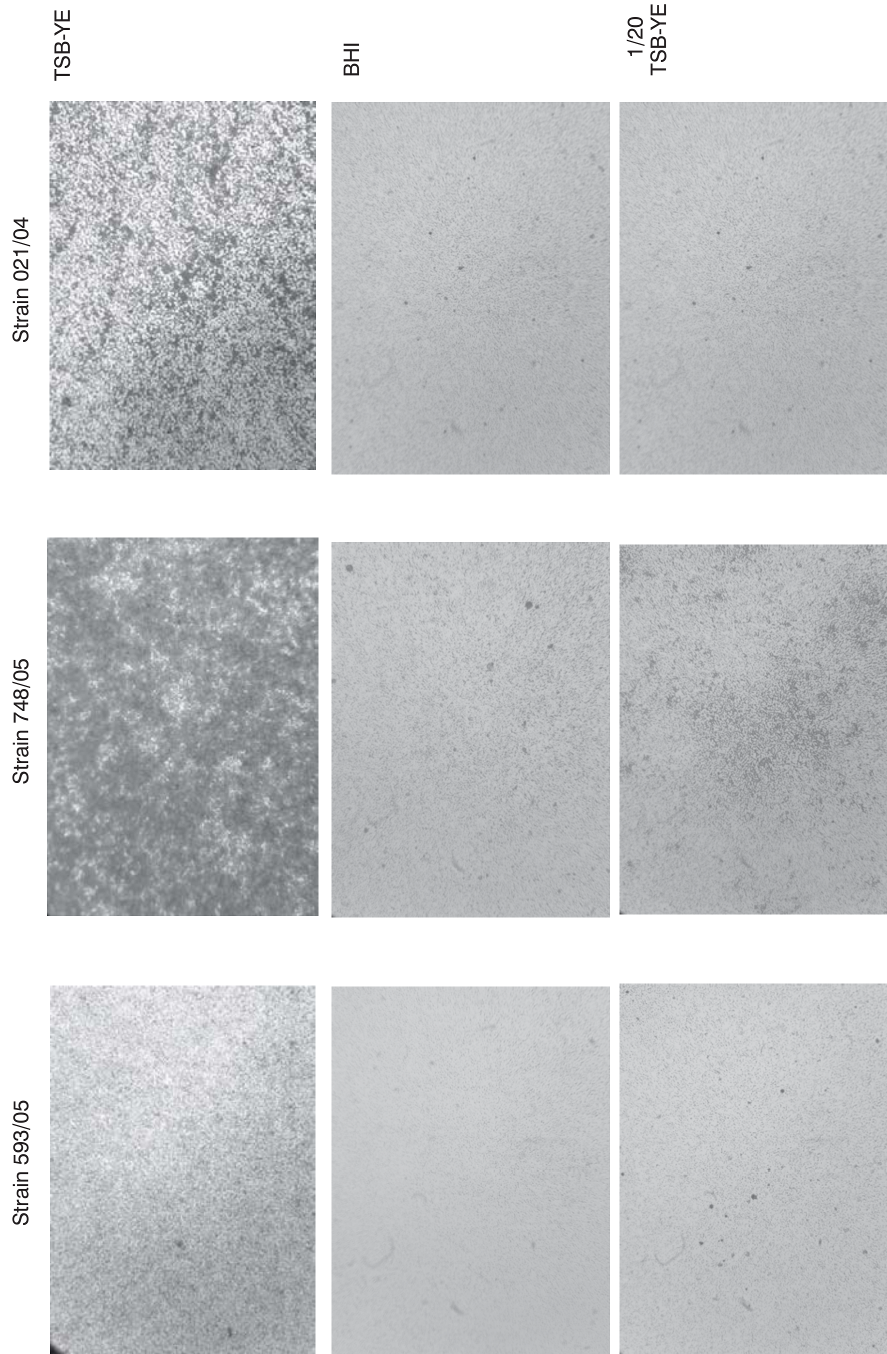

Figure 2. Light microscopy (20x) of *L. monocytogenes* strains 593/05, 748/05 and 021/04 the biofilm on polystyrene surface, at 25ºC, incubation period 48 h, in TSB, BHI and 1/20 TSB

the cell colonies and small cell aggregates the unpopulated surface areas are visible. Considering that only one check-out was made in 48 hours, it is still unclear whether these unpopulated areas were uncolonized or might result from cell detachment. Culturing on BHI at 37°C gives an impression of weaker colonization of the plate in comparison with the TSB-YE, i.e. the unpopulated surface areas between microcolonies in these biofilm networks were much larger.

Higher colonization rate was observed at 25°C on TSB-YE and BHI then at 37°C. The investigated strains are homogenously distributed over the substrate, while strain 748/05 reveals cell aggregates of higher density. At this incubation temperature, culturing in BHI revealed lower growth and colonization rate as compared to TSB-YE. Such microscopic appearance strongly corresponds with the results of the microtiter test, where higher values were obtained on cultivation of *L. monocytogenes* strains in TSB-YE then in BHI. Furthermore, low values obtained in CV test by culturing in 1/20 TSB-YE were confirmed by microscopy, establishing poor growth and colonization of the tested strains in this nutritive medium at both incubation temperatures.

Better colonization rate at 25°C compared to 37°C may well result from the positive influence of the flagella. Flagella play an important role in the initial attachment of bacteria to different substrates, acting as adhesive proteins from one side, and contributing (through their kinetic energy) to overpowering the repulsive forces between electronegative charge of the bacterial cell surface and inert surface. The role of the flagella of *L.monocytogenes* in the initial attachment to stainless steel was established in a study of Vatanyoopaisarn *et al*. (2000).

ACKNOWLEDGEMENT:
This investigation was supported by a grant from the Ministry of Science and the Environmental protection of the Republic of Serbia, Project No. 6860.

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

REFERENCES:

1. *Blackman IC, Frank J,* 1996, Growth of *Listeria monocytogenes* as a biofilm on various food processing surfaces, *J Food Protec*, 59, 8, 827-31.
2. *Borucki K Monica, Peppin JD, White D, Loge F, Call DR,* 2003, Variation in Biofilm Formation among Strains of *Listeria monocytogenes*, *Appl Envir Microbiol*, 69, 12, 7336-42.
3. *Broschat SL, Call DR, Kuhn EA, Loge FJ,* 2005, Comparison of the reflectance and Crystal Violet assays for measurement of biofilm formation by *Enterococcus*, *Biofilms*, 2, 177-81.
4. *Carpentier B, Chassaing D,* 2004, Interactions in biofilms between *Listeria monocytogenes* and resident microorganisms from food industry premises, *Int J Food Microbiol,* 97, 111-22.
5. *Chae MS, Schraft H,* 2000, Comparative evaluation of adhesion and biofilm formation of different *Listeria monocytogenes* strains, *Int J Food Microbiol*, 62, 103-11.
6. *Chavant P, Martinie B, Meylheuc T, Bellon-Fontaine MN, Hebraud M,* 2002, *Listeria monocytogenes* LO28: Surface physicochemical properties and ability to form biofilms at different temperatures and growth phases, *Appl Environ Microbiol*, 68, 2, 728-37.

7. *Chmielewski RAN, Frank JF,* 2003, Biofilm Formation and Control in Food Processing Facilities, *Compr Rev Food Sci Food Saf*, 2, 22-32.
8. *Donlan RM, Costerton JW, 2002,* Biofilms: Survival Mechanisms of Clinically Relevant Microorganisms, *Clin Microbiol Rev*, 15, 2, 167-93.
9. *Đorđević D, Wiedmann M, McLandsborough A,* 2002, Microtiter Plate Assay for Assessment of *Listeria monocytogenes* Biofilm Formation, *Appl Environ Microbiol*, 68, 6, 2950-8.
10. *Harvey J, Keenan KP, Gilmour A,* 2007, Assessing biofilm formation by *Listeria monocytogenes* strains, *Food Microbiol*, 24, 380-92.
11. *Hood S, Zottola E,* 1997, Adherence to stainless steel by food borne microorganisms during growth in model food systems, *Int J Microbiol*, 37, 145-53.
12. *Jeong DK, Frank JF,* 1994, Growth of *Listeria monocytogenes* at 10°C in biofilms with microorganisms isolated from meat and dairy processing environments, *J Food Prot*, 57, 7, 576-86.
13. *Leriche V, Carpentier B,* 2000, Limitation of adhesion and growth of *Listeria monocytogenes* on stainless steel surfaces by *Staphylococcus sciuri* biofilms, *J Appl Microbiol*, 88, 594-605.
14. *Lunden JM, Miettinen MK, Autio TJ, Korkeala HJ,* 2000, Persistent *Listeria monocytogenes* strains show enhanced adherence to food contact surface after short contact time, *J Food Prot*, 63, 1204-7.
15. *Mafu AA, Roy D, Goulet J, Magny P,* 1990, Attachment of *Listeria monocytogenes* to stainless steel, glass, polypropylene and rubber surfaces after short contact time, *J Food Prot*, 53, 742-6.
16. *Marsh EJ, Hongliang L, Wang H,* 2003, A theree-tiered approach to differentiate *Listeria monocytogenes* biofilm-forming abilities, *FEMS Microbiol Lett*, 228, 2, 203-10.
17. Møretrø T, Langsrud S, 2004, *Listeria monocytogenes*: biofilm formation and persistence in food-processing environments, *Biofilms*, 1, 107-21.
18. *Norwood DE, Gilmour A,* 2001, The differential adherence capabilities of two *Listeria monocytogenes* strains in monoculture and multispecies biofilms as a function of temperature, *Lett Appl Microbiol*, 33, 320-4.
19. *Notermans S, Dufrenne J, Teunis P, Chrackraborty T,* 1998, Studies on the risk assessment of *Listeria monocytogenes*, *J Food Prot*, 61, 244-8.
20. *Rönner AB, Wong ACL,* 1993, Biofilm development and sanitizer inactivation of *Listeria monocytogenes* and *Salmonella typhimurium* on stainless steel and Buna-N rubber, *J Food Protec*, 56, 750-8.
21. *Stepanović S, Ćirković I, Ranin L, Švabić-Vlahović M,* 2004, Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surface, *Lett Appl Microbiol*, 38, 428-32.
22. *Vogel BF, Huss HH, Ojeniyi B, Ahrens P, Gram L,* 2001, Elucidation of *Listeria monocytogenes* contamination routes in cold-smoked salmon processing plants detected by DNA-based typing methods, *Appl Environ Microbiol*, 67, 2586-95.
23. *Vatanyoopaisarn S, Nazli A, Dood CER, Rees CED, Waites WM*, 2000, Effect of flagella on initial attachment of *Listeria monocytogenes* to stainless steel, *Appl Environ Microbiol*, 66, 2, 860-3.
24. *Wang H,* 2004, Biofilm development by *Listeria monocytogenes*, Biofilms in food environment: Current approaches and findigs, 2004 IFT Annual Meeting, July 12-16, Las Vegas, NV.

ISPITIVANJE SPOSOBNOSTI FORMIRANJA BIOFILMA U USLOVIMA *IN VITRO* SOJEVA *LISTERIA MONOCYTOGENES* IZOLOVANIH OD ŽIVOTINJA

MILANOV DUBRAVKA, AŠANIN RUŽICA, MIŠIĆ D, VIDIĆ BRANKA i RATAJAC R

SADRŽAJ

Listeria monocytogenes je uzročnik listerioze ljudi i životinja i veoma važan patogen koji pored ostalih načina prenošenja može da se prenese i hranom. Kon-

trola njenog prisustva u pogonima prehrambene industrije, naročito na mestima moguće kontaminacije namirnica, od izuzetnog je značaja u proizvodnji zdravstveno bezbedne hrane i zaštiti zdravlja ljudi. Istraživanja su pokazala da se ova bakterija više meseci ili godina uzastopno može izolovati sa različitih mesta sa kojima u kontakt dolaze namirnice, upravo zahvaljujući sposobnosti da se vezuje za inertne površine i formira biofilm, sama ili u zajednici sa drugim vrstama bakterija.

U ovom radu je ispitivana sposobnost formiranja biofilma u mikrotitracionim pločama od polistirena kod 16 izolovanih sojeva *L. monocytogenes*, poreklom od životinja. Ispitivanja su vršena na temperaturama od 4°C, 25°C i 37°C, na kojima *Listeria monocytogenes* može uobičajeno da raste. Za navedena ispitivanja korišćene su tri hranljive podloge različitog sastava, kao što su tripton soja bujon sa kvaščevim ekstraktom (TSB-YE), moždano srčana infuzija (BHI) i tripton soja bujon sa kvaščevim ekstraktom razređen u odnosu 1:20 fiziološkim rastvorom (1/20 TSB-YE). Za ispitivanja formiranja biofilma in vitro pripreman je inokulum od kultura izolovanih sojeva *L. monocytogenes* starih 24 časa. Gustina pripremljenog inokuluma iznosila je 2-10x10^7 CFU/ml (OD_{600}=0,093 ± 0,009 u TSB-YE). Mikrotitracione ploče nakon inokulisanja inkubisane su tokom 48 časova na prethodno navedenim temperaturama. Kolonizacija sojeva *L. monocytogenes* na polistiren i formiranje biofilma, praćeno je pomoću kristal violet boje koja je dodavana u mikrotitracione ploče, kao i pomoću svetlosnog mikroskopa. Sojevi *Listeria monocytogenes* koji su ispitivani pokazali su različitu sposobnost formiranja biofilma u zavisnosti od temperature inkubacije i podloge koja je korišćena za rast. Zbog navedenih činjenica u radu su prikazani rezultati dobijeni ispitivanjem četiri soja *Listeria monocytogenes* koji su izolovani iz uzoraka mozga obolelih ovaca i označeni brojevima 785/05, 593/05, 748/05, 1915/04 i jedan soj izolovan iz pobačenog fetusa krave označen brojem 021/04, kao i jednog referentnog soja *L. monocytogenes* 1071 (4b) koji je korišćen kao kontrola. Sojevi *L. monocytogenes* koji su bili zasejavani u podloge bogatije hranljivim materijama, kao što je (TSB-YE) i kultivisani na višim temperaturama (na temperaturi od 37 °C) bolje su formirali biofilm.

Nijedan od ispitivanih sojeva nije mogao biti procenjen kao jak biofilm "producer", bez obzira na temperaturu inkubacije ili korišćenu podlogu. Najviše OD vrednosti dobijene su na temperaturi inkubacije od 37°C u TSB-YE (OD_{595}=0,346 ±0,055), kada su tri ispitana soja kategorisana kao umereni, a dva kao slabi produktori biofilma. Sa smanjenjem temperature inkubacije u istoj podlozi, smanjivale su se i OD vrednosti, pa su na temperaturi od 25°C (OD_{595}=0,289 ± 0,083) četiri soja procenjena kao slabi biofilm, a na temperaturi od 4°C (OD_{595}=0,124 ± 0,011) nijedan soj nije procenjen kao produktor biofilma. Kvantitativne vrednosti dobijene primenom mikrotitracionog testa sa kristal violet bojom, koji se pokazao kao jednostavna i brza (skrining) metoda za procenu sposobnosti sojeva *L. monocytogenes* da formiraju biofilm pod različitim uslovima, potvrđene su i primenom svetlosne mikroskopije.