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## Distribution of extracellular DNA in *Listeria monocytogenes* biofilm

MARTINA ŠULÁKOVÁ<sup>1\*</sup>, JARMILA PAZLAROVÁ<sup>1</sup>, RIKKE LOUISE MEYER<sup>2</sup>,  
KATEŘINA DEMNEROVÁ<sup>1</sup>

<sup>1</sup>Department of Biochemistry and Microbiology, Faculty of Food and Biochemical Technology, UCT Prague, Czech Republic

<sup>2</sup>Interdisciplinary Nanoscience Center – INANO-Bioscience, iNANO-huset, Aarhus University, Denmark

\*Corresponding author: [martina.bohacova@vscht.cz](mailto:martina.bohacova@vscht.cz)

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**Abstract:** Extracellular DNA (eDNA) is an abundant matrix component that protects biofilm from environmental stress, facilitate horizontal gene transfer, and serve as a source of nutrients. eDNA is also found in *Listeria monocytogenes* biofilm, but it is unknown to which extent its importance as a matrix component varies in terms of phylogenetic relatedness. This study aims to determine if these variations exist. Biofilm forming capacity of ten *L. monocytogenes* strains of different phylogenetic lineages and serotypes was examined using crystal violet assay at 37°C and 22°C. eDNA content was evaluated fluorometrically at 37°C and at 22°C, then the 3D structure of biofilm was studied by confocal laser scanning microscopy (CLSM). Biofilm forming capacity differed significantly between the culturing conditions and was higher at 37°C than at ambient temperature. eDNA signal distribution was found to be influenced by strain and lineage. CLSM images revealed information about spatial distribution in the biofilm. The information about the eDNA spatial organisation in the biofilm contributes to the understanding of the role of eDNA in a biofilm formation.

**Keywords:** biofilm; extracellular DNA; *Listeria*; confocal laser scanning microscopy

Biofilms are structured communities of cells engulfed in self-produced polymeric matrix that adheres to biotic and abiotic surfaces (COSTERTON 1999; DONELLI *et al.* 2010) biofilm phenotype conveys resistance to various stress factors that are present in the environment (DEVAUX & HEBRAUD 2006; CARPENTIER & CERF 2011). Therefore, its presence has implications in the food industry, modern technologies like bioremediation processes as well as in human health (KOKARE *et al.* 2009).

The biofilm matrix is composed of extracellular polymeric substances (EPS), such as proteins, polysaccharides and extracellular DNA (eDNA). Its composition is species-specific and highly influenced by the external environment. The matrix is crucial for adhesion, colo-

nisation, and nutrient capture, and it is the site of horizontal gene transfer (FLEMMING 2011). The biofilm matrix also provides a protective barrier between the cells and the surrounding environment (SUTHERLAND 2001; BRANDA *et al.* 2005; FLEMMING & WINGENDER 2010). It has been suggested that resistance to biocides is affected by the composition of the matrix, and not only by the cells themselves (PAN *et al.* 2006), as the matrix decreases the penetration of some antimicrobials (FLEMMING & WINGENDER 2010). Importantly, the polymeric substance producers have an evolutionary advantage in competition among the strains that do not produce much EPS, as the producers suffocated their growth (XAVIER & FOSTER 2007).

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eDNA conserved molecule that is present in the biofilm matrix of bacteria across the phylogenetic lineages and it is also ubiquitous in the environment (OKSHEVSKY & MEYER 2015; BOHÁČOVÁ *et al.* 2018; NAGLER *et al.* 2018). However, its quantity varies strongly from species to species, and even among closely related strains (IZANO *et al.* 2008). Production of the eDNA is often associated with cell death, autolysis, and secretion (DE ALDECOA *et al.* 2017). eDNA can serve as site and reservoir for horizontal gene transfer, but equally important is its role in adhesion and coaggregation of bacteria, and while stabilizing the physical structure of biofilms (FLEMMING & WINGENDER 2010; JAKUBOVICS *et al.* 2013). eDNA also conveys protection against positively charged antimicrobials (DELL'ANNO & DANOVARO 2005; CHIANG *et al.* 2013; JAKUBOVICS *et al.* 2013).

The apparent ubiquity of eDNA in biofilms and its role in biofilm-assisted antibiotic resistance makes eDNA an interesting target for anti-biofilm therapies (OKSHEVSKY *et al.* 2015; BOHÁČOVÁ & PAZLAROVÁ 2018). Therefore, it is important to have a detailed understanding of how the role of the eDNA in biofilm formation and resilience varies among bacterial strains, and how it is affected by the conditions in which the biofilm was grown.

*Listeria monocytogenes* is a biofilm forming, ubiquitous, and opportunistic pathogen associated with food. It causes infection in vulnerable groups of the population such as the elderly, immunocompromised individuals, pregnant women and newborns (FARBER & PETERKIN 1991; VÁZQUEZ-BOLAND *et al.* 2001). *L. monocytogenes* have been shown sensitive to the eDNA disruption (OKSHEVSKY & MEYER 2013).

As the matrix eDNA of *L. monocytogenes* biofilm exhibit several crucial structural functions, we characterise its distribution and quantity in strains with a known phylogenetic background. The capacity to

form biofilm was assessed in two different growth conditions, at ambient temperature mostly occurring in the food processing at 22°C and *in vivo*-like conditions at 37°C. As the characterisation of the eDNA in *L. monocytogenes* has not yet been performed in detail, we assessed the biofilm formation and eDNA release of *L. monocytogenes* strains of different phylogenetic relatedness. In addition, we aim to determine some spatial characteristics of *L. monocytogenes* biofilm.

## MATERIAL AND METHODS

**Strain selection.** The strains of *L. monocytogenes* were obtained from strain collection of the Testing Laboratory of the Department of Biochemistry and Microbiology (UCT Prague). The strain selection was based on the serotype, the lineage, source and year of isolation. The strains were selected from representative serotypes 1/2a, 1/2b, 1/2c, 4b. All strains originated in the Czech Republic. The list of strains representing each serotype is shown in Table 1.

**Growth and storage conditions.** Bacterial cultures were stored at –80°C in Tryptic Soy Broth (TSB) (Merck KGaA, Germany) with 25% glycerol. The selected strains were grown on several selective media on ALOA<sup>®</sup> agar (bioMérieux CZ, Czech Republic) for 24–48 h at 37 ± 2°C and stored in the fridge for up to 1 month. One colony was used to inoculate 50 ml of Brain Heart Infusion broth (Merck KGaA, Germany) and incubated at 37 ± 2°C overnight with shaking at 120 rpm. The overnight cultures were then used to inoculate in TSB. Alternatively, the overnight cultures were diluted to OD<sub>600</sub> = 0.1 in 1/10 TSB, and 200 µl was transferred to each well in microtiter plates (COSTAR Corning, USA) and incubated under static conditions for 24 ± 2 h at ambient temperature, approximately

Table 1. The list of *L. monocytogenes* strains.

Strain number	Source	Phylogenetic lineage	Serotype	Year of isolation
LM 77	RTE food – raw meat	I	4b	2005
LM 96	RTE food – delicacy	I	1/2b	2006
LM 119	raw food – raw meat	II	1/2c	2007
LM 129	human isolate – sporadic case	II	1/2a	2007
LM 131	raw food – raw meat	II	1/2c	2007
LM 147	RTE food – raw meat	I	4b	2007
LM 156	RTE food – dairy	II	1/2a	2008
LM 164	RTE food – raw meat	I	1/2b	2008
LM 165	RTE food – raw meat	II	1/2a	2008
EGD-e	animal	II	1/2a	–

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22 ± 2°C. Half of the supernatant (100 µl) was discarded the next day, planktonic cells were removed by two washing steps using 200 µl phosphate buffered saline (Merck KGaA, Germany), pH = 7.4.

**Crystal violet assay.** The overnight cultures were adjusted to reach OD<sub>600</sub> = 0.1 in TSB. Next, 200 µl of the suspension was transferred into each well in microtitre plates Nunc 96 Catalog No. 163320 (Merck KGaA, Germany) and incubated under static conditions for 24 ± 2 h at 37°C or 22 ± 2°C. The biofilm forming capacity was assessed using a crystal violet assay. The protocol was adopted from DJORDJEVIC *et al.* (2002). The biofilm was stained by 100 µl 0.1% crystal violet solution for 30 min. The biofilm was washed three times with sterile distilled water. The dye was released using 100 µl 96% ethanol. The absorbance was measured using Biotek plate reader Powerwave XS2 (BioTek Instruments, Inc., USA) at 595 nm 15 min after the application of ethanol. The data were analysed Statistica 13.1 software. Three independent replicates were used for statistical analysis.

**Fluorimetry.** The PicoGreen® staining solution Quant-iT™ PicoGreen® dsDNA Reagent (Invitrogen TM, USA) was diluted 1/200 in Tris-EDTA buffer solution and mixed with the sample in ratio (1 : 1) (TANG *et al.* 2013). Fluorescence was measured using a PerkinElmer 2030 plate reader (PerkinElmer Inc.; USA) ( $\lambda_{\text{ex}} = 485$ ,  $\lambda_{\text{em}} = 535$  nm) within 10 min after the addition of the dye. Salmon sperm dsDNA (Sigma-Aldrich, Germany) was used as a standard for calibration.

**Confocal laser scanning microscopy.** eDNA was stained using cell impermeable TOTO®-1 iodide (Invitrogen TM, USA) to a final concentration 2 µM. DNA of intact cells was stained by cell-permeable SYTO 60® (Invitrogen TM, USA) to final concentration 10 µM (OKSHEVSKY & MEYER 2014). Three images were captured from each well, using a Zeiss 700 confocal laser scanning microscope equipped with objective Alpha plan Apochromat 100x/1.46 Oil (Zeiss, Switzerland), and excitation of the fluorophores by lasers 488 nm (TOTO®-1) and 635 nm (SYTO 60®). Images in z-stacks were acquired in 0.4 µm steps. Projections and section of biofilm were generated using ZEN 2012 blue edition software (Zeiss, Switzerland).

**Image analysis.** Quantitative analysis of 3D images was performed by COMSTAT software (HEYDORN *et al.* 2000). Several parameters, including biovolume that represents biomass, surface exposed to nutrients and roughness coefficient that represents variability in height of the biofilm, were observed for each strain under both conditions.

**Data analysis.** All outputs were evaluated in Statistica 13.1. All evaluated data were based at least on triplicates. The data are expressed as mean ± standard deviation (s.d.). Following codes were used to describe statistical significance (\*). The  $P < 0.05$  were considered statistically significant. Primarily, the normality was tested using Anderson-Darling test. Then, the variables (biofilm formation, eDNA concentration, ratios) were analysed using MANOVA. If applicable, Tukey HSD test was used to evaluate inter-stains, inter-lineages differences.

## RESULTS AND DISCUSSION

We observed the impact of culturing conditions on *L. monocytogenes* biofilm and its eDNA. Biofilm forming capacity of 10 tested strains was assayed using crystal violet. The growth conditions strongly affected the amount of biofilm formed, as biofilm formation doubled when grown in full-strength TSB and at 37°C ( $P < 0.05$ ). Significantly more biofilm was formed in high nutrient environment at 37°C than at ambient temperature in low nutrient media (Figure 1). PAN *et al.* (2010) showed a similar effect for TSB enriched with 0.6% yeast extract at 22.5 and 37°C. Our study confirmed the results of previous groups who have demonstrated the impact of culturing conditions on biofilm formation of *L. monocytogenes* (DJORDJEVIC *et al.* 2002; PAN *et al.* 2010; COMBROUSE *et al.* 2013; KADAM *et al.* 2013). Biofilm formation of strains differed significantly among the strains in both culturing conditions ( $P < 0.001$ ). Additionally, the strains of lineage I formed significantly more biofilm than strains from the lineage II ( $P < 0.05$ ). This difference was observed at 37°C, but not at 22°C. Our results support the findings of DJORDJEVIC *et al.* (2002) who also observed increased biofilm formation for strains of lineage I. In contrast to these results were the observation of BORUCKI *et al.* (2003), but both authors used slightly different culturing conditions for biofilm development. Notably, the experimental design of DJORDJEVIC *et al.* (2002) was more similar to ours.

The eDNA concentration in the biofilms differed significantly between the strains in both conditions ( $P < 0.001$ ), (Figure 2). Our estimated concentration were somewhat higher than those reported for different freshwater species TANG *et al.* (2013) but comparable with those reported previously for *Listeria monocytogenes* in BOHÁČOVÁ *et al.* (2019). Among

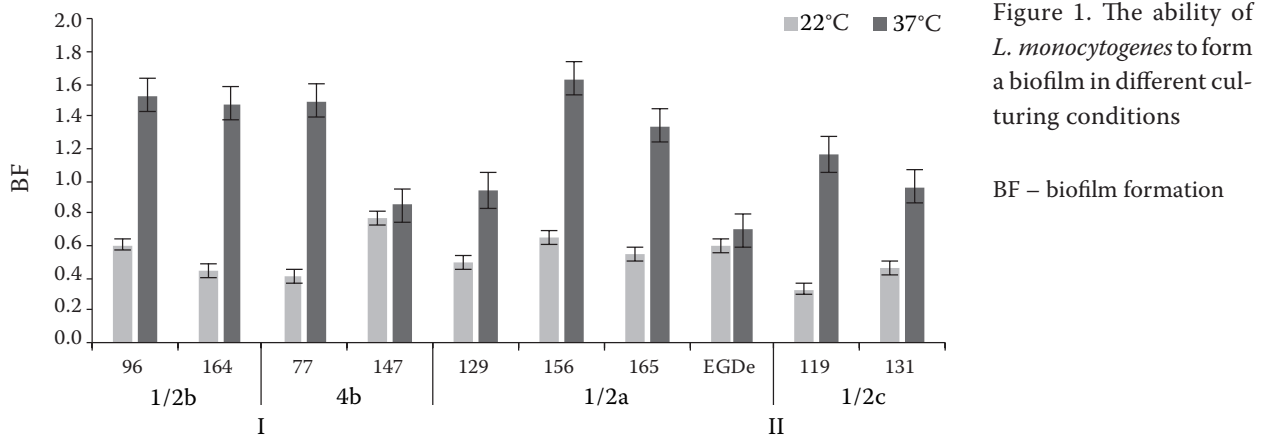


Figure 1. The ability of *L. monocytogenes* to form a biofilm in different culturing conditions

BF – biofilm formation

the growth conditions used in our study, we found the accumulation of the most eDNA at 37°C (Figure 2).

This could be associated with increased growth at 37°C. To eliminate the difference in growth, we linked the amount of eDNA to biofilm formation while creating its ratio (eDNA/biofilm formation ratio).

Interestingly, the members of lineage II had slightly higher eDNA/biofilm formation ratio than strains

from lineage I in both conditions, but the significant increase was observed only at 37°C ( $P < 0.001$ ), Figure 3. Although we observed some differences in eDNA release among phylogenetic lineages, more extensive study would be required to confirm this finding as our samples size is limited. Interestingly, the members of this lineage, mainly of the serotype 1/2a, belong amongst most frequently found isolates in food and also humans in the Czech Republic and amongst most frequently isolated serotypes causing disease worldwide (ORSI et al. 2011; GELBICOVA & KARPISKOVA 2012). Many countries report strains of serotype 4b as more frequent in human listeriosis cases.

Our results confirm the results of ZETZMANN *et al.* (2015) who suggested condition-specific eDNA release based on the action DNase I. To confirm our findings, we used the alternative method for the assessment of eDNA content in biofilm independently. We observed the detailed structure of *L. monocytogenes* biofilm using confocal laser scanning microscopy imaging (CLSM), Figure 4. The channels facilitating nutrient exchange were observed along with eDNA pockets. The eDNA

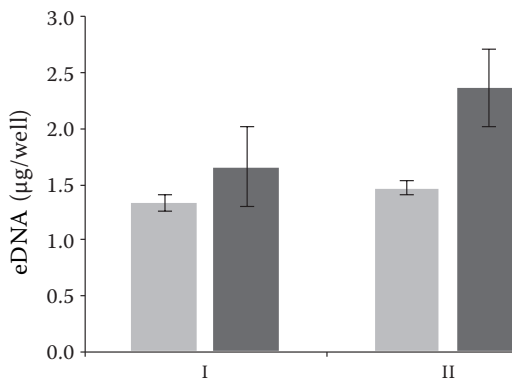


Figure 2. The eDNA content in *L. monocytogenes* biofilm

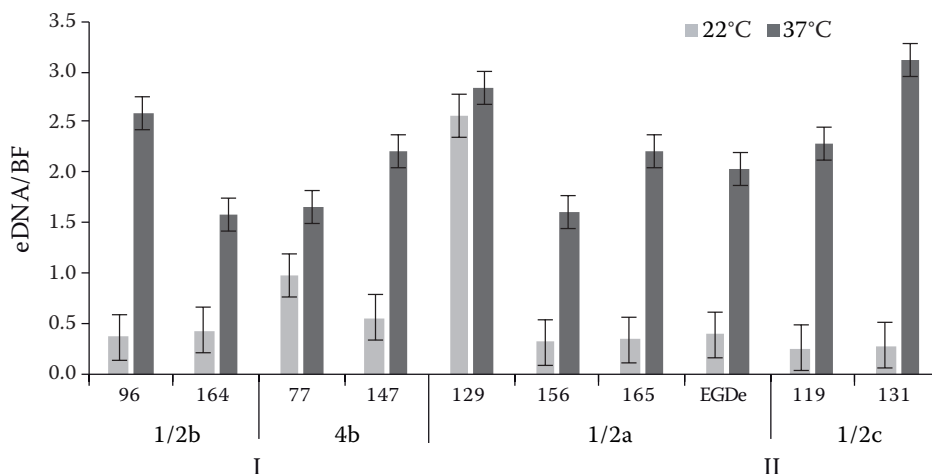


Figure 3. The relationship in eDNA concentrations among 2 phylogenetic lineages of *L. monocytogenes* biofilm standardized per biofilm formation

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and dead cells fulfilled the voids as observed in GUILBAUD *et al.* (2015) in which diversity of biofilms of *L. monocytogenes* was analysed. Additionally, we observed large diffused patches at 22°C similar to those observed in ZETZMANN *et al.* (2015), flatter biofilms

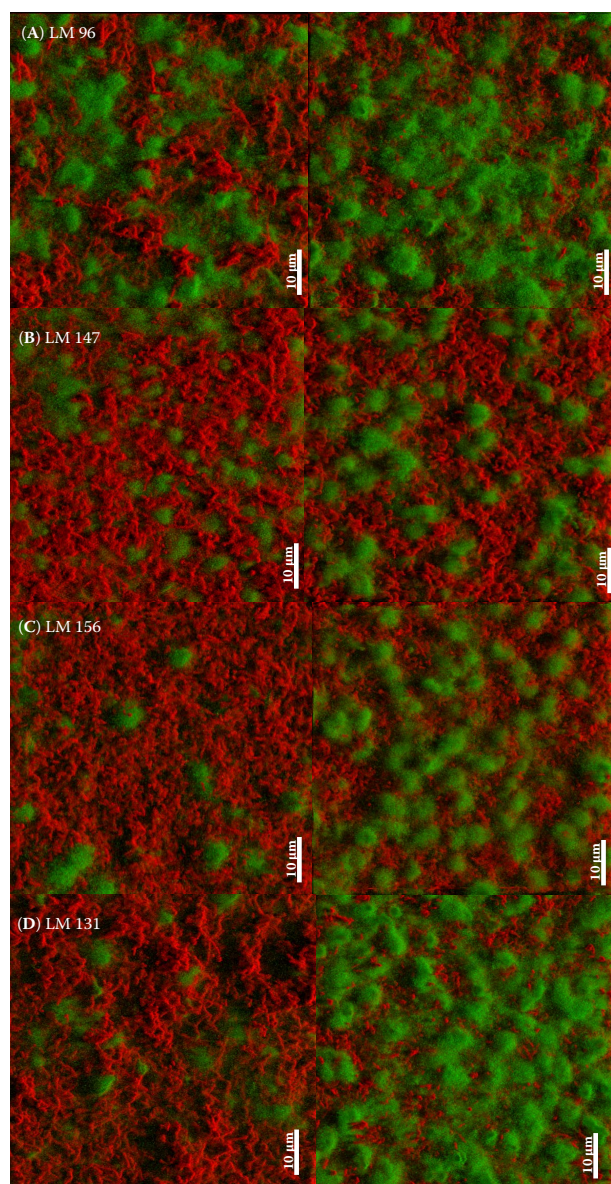


Figure 4. eDNA distribution in selected strains of *Listeria monocytogenes*

left: at 22 ± 2°C temperature in low nutrient environment; right: at 37°C in high nutrient environment; 64 μm × 64 μm; TOTO<sup>®</sup>-1 IODIDE eDNA and death cells; SYTO 60<sup>®</sup> (red) cells; (A) LM 96, phylogenetic lineage I, serotype 1/2b; (B) LM 147, phylogenetic lineage I, serotype 4b; (C) LM 156, phylogenetic lineage II, serotype 1/2a, (D) LM 131, phylogenetic lineage II, serotype 1/2c

were also seen at 37°C, but eDNA content varied between the strains on the top of the biofilm (Figure 4).

The analysis of 3D images facilitated quantification of biomass for each strain (Figure 5). The average biovolume reached 29.44 ± 4.47 μm<sup>3</sup>/μm<sup>2</sup> at 22°C and 32.05 ± 6.36 μm<sup>3</sup>/μm<sup>2</sup> at 37°C. Other authors used biovolume for biofilm characterisation, their values were in similar ranges but used for different microorganisms (XIAO & KOO 2010; DOGHRI *et al.* 2015; PADOVANI *et al.* 2015; POWELL *et al.* 2018). We attempted to capture the images stacks of similar heights. The average biofilm thickness reached 19 ± 3 μm at 22°C and 18 ± 3 μm at 37°C. These observations are supported by the roughness coefficient of biofilm cells that was similar in both culturing conditions (Figure 6). The average roughness coefficient for a signal from eDNA was higher at 22°C than at 37°C, therefore, the eDNA signal pattern within a biofilm was more heterogeneous. These observations agree with Heydorn *et al.* (2000) in which changes in roughness coefficient were observed for *Pseudomonas* spp. biofilm. Additionally, surface exposed to nutrients of the cells was similar in all conditions, perhaps, because of the experimental design. However, the surface exposed to nutrients of eDNA was larger in 22°C, in a low nutrient environment than at 37°C (Figure 7). More eDNA was exposed to nutrients as the biofilm was more heterogeneous and structured, more voids were present (Figure 4). Sticky nature of eDNA can contribute to the interactions with other components, including nutrients, or can be utilized as a nutrient source itself (FINKEL & KOLTER 2001).

It is important to note that eDNA production is influenced by other factors, such as biomass, thickness of the biofilm etc. If other factors are considered, final proportion of eDNA to living cells may be found higher at ambient temperature in low nutrients media as the biofilm is more heterogeneous and surface exposed to nutrients may be higher.

## CONCLUSIONS

Our results supported the observation of the impact of culture conditions on biofilm formation of *L. monocytogenes*. Moreover, the results suggest a boarder role of eDNA in biofilm formation from the perspective of phylogenetic relatedness. Future studies with larger sample size can confirm our findings and explain in detail the impact of phylogeny in eDNA content of the biofilm. The power and usefulness

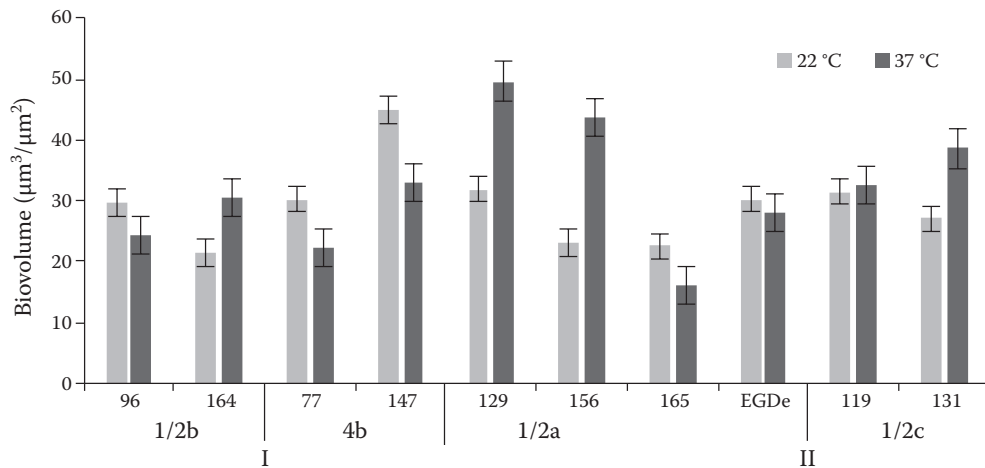


Figure 5. The biomass of *L. monocytogenes* biofilm in different culturing conditions

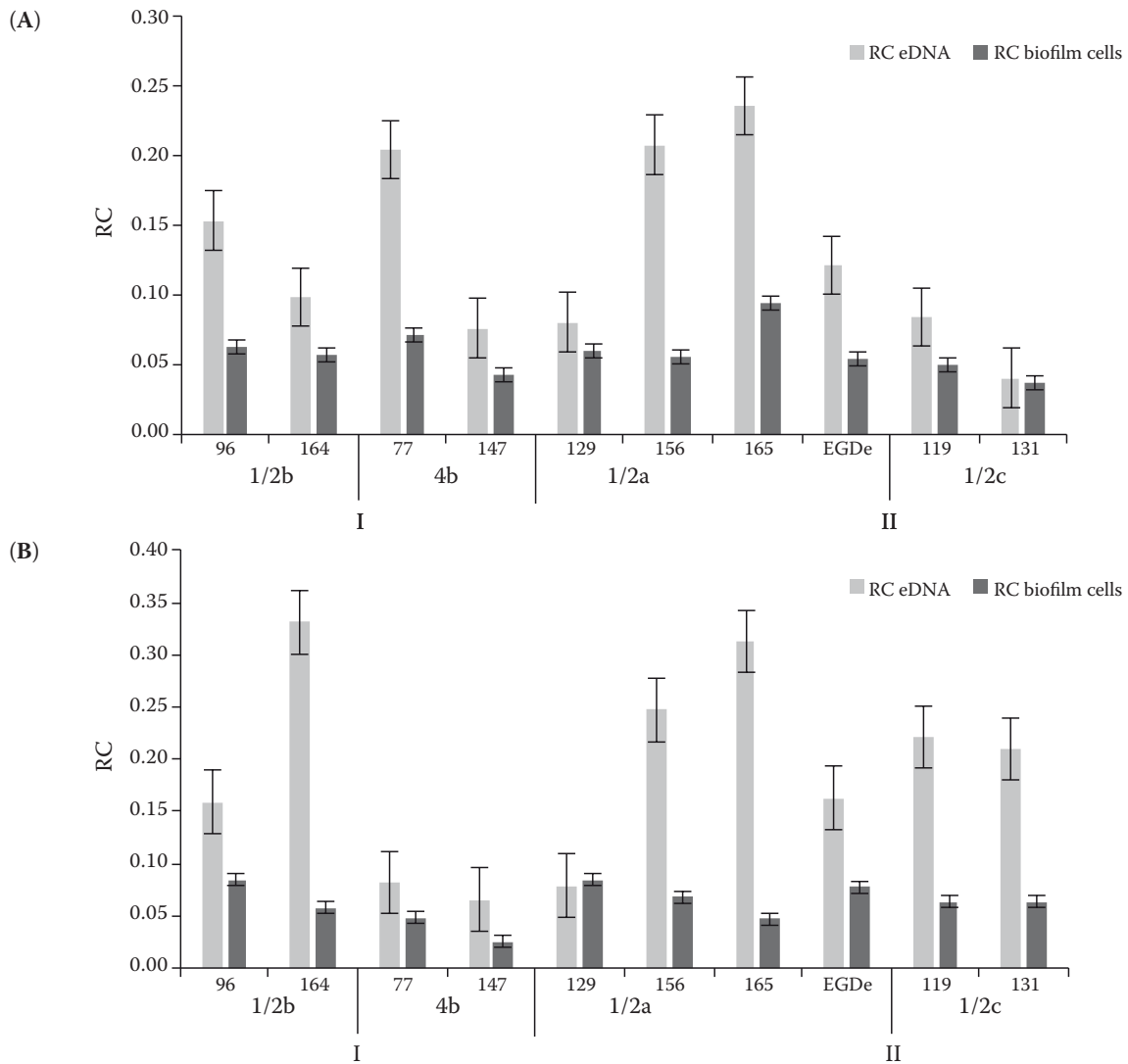


Figure 6. The heterogeneity of *L. monocytogenes* biofilm

(A) 37°C; (B) 22°C; RC– roughness coefficient

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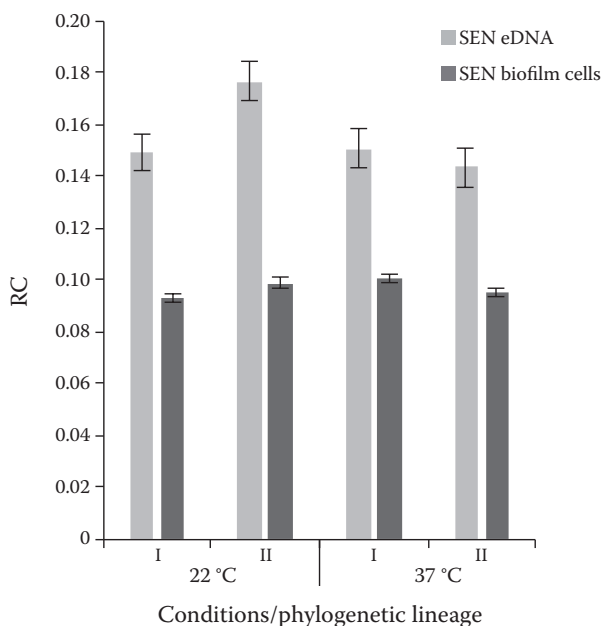


Figure 7. Biofilm surface exposed to nutrients of *L. monocytogenes* in different culturing conditions

SEN – surface exposed to nutrients; RC – roughness coefficient

of the modern imaging techniques and image analysis were demonstrated, as the quantitative analysis of 3D image can give more detailed information about a spatial structure of the biofilm and matrix interactions.

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