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The effect of blood on the ability of biofilm formation by *Listeria monocytogenes* strains

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ABSTRACT

Introduction: *Listeria monocytogenes* strains are widespread in the natural environment and are the etiological factor of listeriosis. Food is the main source of *L. monocytogenes*. Secondary contamination of food products which results from the formation of biofilm by *L. monocytogenes* on the surfaces of the processing devices makes an essential problem.

Material and methods: We evaluated the ability of biofilm formation in media supplemented with sheep blood (in a volume of 5.0, 10.0, 20.0 and 50.0%) for the reference strain *L. monocytogenes* ATCC 7644 and strains isolated from the human blood and carrots on the polypropylene surface.

Results: The strain isolated from blood most efficiently formed a biofilm with increasing blood sheep's addition (nutrient broth — 4.87×10^5 CFU \times cm², blood supplement 50.0% (v/v) — 3.4×10^7 CFU \times cm²). The number of *L. monocytogenes* recovered from the biofilm for the reference strain and the strain from carrot increased to 20.0% by volume of the blood addition (3.07×10^5 CFU \times cm² and 4.03×10^5 CFU \times cm² respectively — nutrient broth; 1.1×10^7 CFU \times cm² and 9.23×10^6 CFU \times cm² — blood supplement 20.0% (v/v)). Decrease in the number of cells recovered from the biofilm for the reference strain and the one isolated from carrot has been demonstrated at 50.0% addition of blood into nutrient medium (8.8×10^6 CFU \times cm² and 7.87×10^6 CFU \times cm² respectively).

Conclusion: The addition of sheep blood to the medium at concentrations up to 20.0% increases the number of cells recovered from the biofilm for all studied *L. monocytogenes* strains whereas medium with 50.0% blood stimulates the biofilm formation only by the strain isolated from blood.

Key words: biofilm, *Listeria monocytogenes*, nutrient broth, blood, polypropylene

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Introduction

Listeria monocytogenes are gram-positive, relatively anaerobic, non-enveloped and non-spore-forming bacilli, commonly found in the natural environment (water, soil, sewage, decaying vegetation or silage) [1]. These bacteria cause listeriosis, dangerous for pregnant women and newborns as well as elderly people [2]. Food, including raw meat, fish (fresh and smoked), vegetables, fruits and dairy products are their main source [3]. Secondary contamination of food products may be the result of biofilm formation by *L. monocytogenes* on the surfaces of equipment used in food processing plants [4–5]. *L. monocytogenes* strains are capable of colonizing various surfaces contacting with food,

including polystyrene, polypropylene, glass, stainless steel, quartz, marble and granite [6]. Biofilm formation is induced by many environmental factors found in the food processing industry, such as: pH changes, increased oxygen concentration, and also depends on the availability of nutrients

[7–8]. Limited access to nutrients induces stress in *L. monocytogenes*, resulting in increased biofilm formation [7–9]. It has been shown that the presence of food production by-products (meat juice, pork serum or fat) in the processing environment stimulates the biofilm formation [10]. Conversion of planktonic forms to the mature biofilm structure increases the chance of survival of *L. monocytogenes* and determines the acquisition of resistance to environmental factors (UV radiation,

acids) and antimicrobial compounds, including antibiotics, which makes it difficult to eliminate biofilms in the food processing plants [7–8]. Extracellular polymeric substances (EPS) protect the cells of bacteria in the biofilm from the unfavorable factors of the processing environment. EPS is composed of protein, DNA, polysaccharides and other extracellular compounds. EPS mediates attachment of *L. monocytogenes* to the surface and provides mechanical stability to the formed biofilm [6].

The aim of study was to assess the ability of biofilm formation by *L. monocytogenes* strains on the polypropylene surface in a nutrient medium enriched with sheep blood.

Materials and methods

Material for research

The reference *Listeria monocytogenes* ATCC 7644 strain and two strains of these species isolated from human blood and carrots, from the collection of the Department of Microbiology, L. Rydygier Collegium Medicum in Bydgoszcz, were used for the study. Isolates were stored at -80°C in BHI (Brain Heart Infusion) medium (bioMérieux) with the addition of 15.0% glycerol.

Strains preparation

In the first stage culture of deep-frozen strains on Columbia Agar with 5.0% sheep blood (bioMérieux) was used. After incubation at 37°C for 24 hours, a passage of the grown colonies of the tested strains on the same type of substrate was performed. The obtained colonies of *L. monocytogenes* were used in the subsequent studies.

Assessment of biofilm formation by *L. monocytogenes* on polypropylene surface in nutrient medium enriched with sheep blood

Sterile polypropylene plates were used in the experiments. The culture medium was prepared by mixing sterile nutrient broth (Merck) with sheep blood. The blood supplement was 5.0, 10.0, 20.0 and 50.0% of the volume (v/v). The nutrient broth without addition of sheep blood was used as a control.

Suspensions of examined strains (0.5 McFarland standard) were prepared in 3 ml of phosphate-buffered physiological saline (PBS) (Avantor). Then suspensions were centrifuged for 5 minutes (3000 rpm) and the supernatants removed. The previously prepared medium was added to the test tubes with sediment, mixed for

2 minutes and added on the polypropylene plates that were incubated at 37°C for 24 hours. After the incubation, the plates were removed and washed with PBS. Plates with the biofilm of *L. monocytogenes* strains were placed in test tubes containing 3 ml of PBS solution and sonicated for 10 minutes (30 kHz, 150W). A series of decimal dilutions in PBS was made and culture was made on Columbia Agar with 5.0% sheep blood. The experiment was performed in triplicate for each tested strain. After the incubation period (24 hours, 37°C), the grown colonies were counted and expressed as the number of colony forming units per cm^2 ($\text{CFU} \times \text{cm}^{-2}$).

Statistical analysis

The results were subjected to statistical analysis with the use of Statistica 12.0 PL (StatSoft). For each strain, the median number of bacteria recovered from the biofilm was calculated in each variant of the experiment. Statistically significant differences between them were evaluated based on the Bonferroni post-hoc test at the significance level of 0.05.

Results

All tested strains of *L. monocytogenes* formed a biofilm, both in the medium with and without the addition of sheep blood (Fig. 1).

In the medium without blood addition (control) the number of cells recovered from the biofilm ranged from $3.07 \times 10^5 \text{ CFU} \times \text{cm}^{-2}$, for the reference *L. monocytogenes* ATCC 7644 strain, to $4.87 \times 10^5 \text{ CFU} \times \text{cm}^{-2}$ for the human blood isolated strain and $4.03 \times 10^5 \text{ CFU} \times \text{cm}^{-2}$ for a carrot isolated strain (Fig. 1). The number of cells recovered for all tested strains, in the medium without the addition of blood did not differ significantly (Fig. 1).

The addition of sheep blood in the volume of 5.0, 10.0 and 20.0% to the medium, stimulated the tested strains of *L. monocytogenes* to biofilm formation. For each of them, The number of cells recovered from the biofilm was significantly higher at each subsequent blood addition to the medium (Fig. 1).

In a medium with the highest content of blood the number of cells recovered from the biofilm increased from $9.23 \times 10^6 \text{ CFU} \times \text{cm}^{-2}$ for strain derived from carrots and up to $1.40 \times 10^7 \text{ CFU} \times \text{cm}^{-2}$ for the strain isolated from human blood (Fig. 1).

The increase of blood volume in the medium to 50.0% resulted in a significant decrease in the number of biofilm cells recovered for the reference *L. monocytogenes* strains and the carrot isolates (Fig. 1). Under this condition only the strain derived from the human blood, strongly formed the biofilm ($3.40 \times 10^7 \text{ CFU} \times \text{cm}^{-2}$).

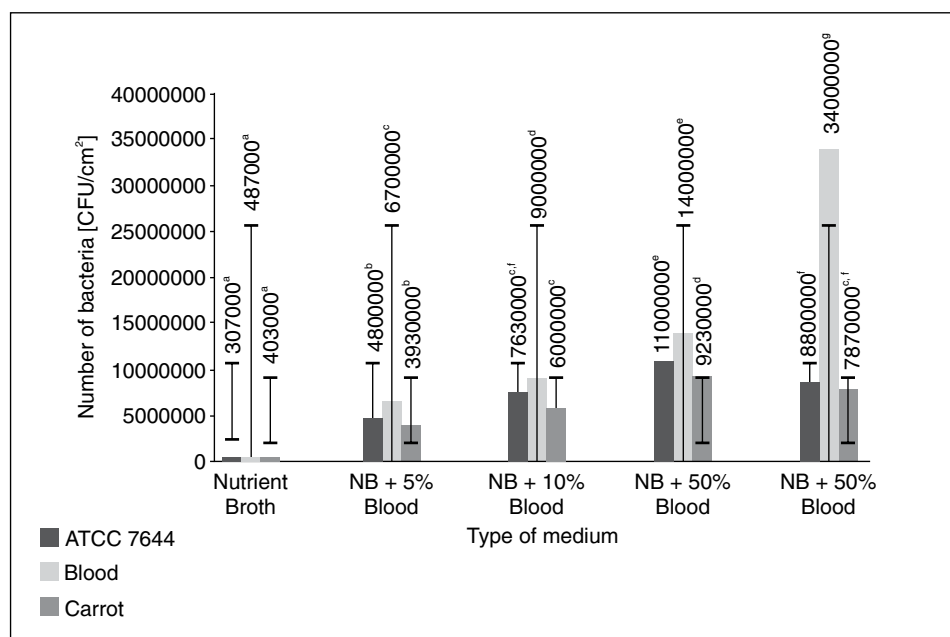


Figure 1. The effect of sheep blood addition on the ability of biofilm formation on the polypropylene surface by *L. monocytogenes* (ATCC 7644 — reference strain; Blood — *L. monocytogenes* strain isolated from blood; Carrot — *L. monocytogenes* strain isolated from carrot). Values marked a, b, c differ significantly.

Regardless of the volumetric addition of blood in the medium, *L. monocytogenes* isolated from blood formed the strongest biofilm whereas the weakest biofilm was formed by the strain isolated from carrots the weakest (Fig. 1).

Discussion

Biofilm formation by *L. monocytogenes* is common, but undesirable, especially in the food industry. These strains in the biofilm structure may be a source of secondary food contamination. The conventional disinfection methods are less effective in eliminating this structure. Many factors influence the development of biofilm, including the availability of nutrients, temperature and pH.

In this study, the ability to form biofilm by three *L. monocytogenes* strains: isolated from human blood, from carrots and the *L. monocytogenes* ATCC 7644 reference strain, on the polypropylene surface in the presence of increasing addition of sheep blood to the medium was evaluated. The addition of sheep blood in a volume of 5.0, 10.0 and 20.0% was found to influence the increase in the number of cells recovered from the biofilm for all tested strains. However, the addition of blood in a volume of 50.0% caused a decrease in biofilm formation in the case of the reference strain and strain isolated from carrots. The highest cell recovery

of bacteria from the biofilm for the strain isolated from the human blood has been demonstrated. Di Xia et al. [11] investigated the effect of human plasma on biofilm formation by *Streptococcus agalactiae*, *Streptococcus pyogenes* and *Staphylococcus aureus*. They assessed the impact of 15.0% human plasma and 1.0% of glucose in the nutrient medium on the ability to form biofilms. It was shown that all strains of *S. agalactiae* form a biofilm in the broth supplemented with human plasma, while the strains of *S. aureus* and *S. pyogenes* under these conditions do not form a biofilm structure. The authors found that human plasma rich in proteins and fatty acids is used by *S. agalactiae* which results in changes in the composition of bacterial shields [11]. Among other factors influencing the ability of biofilm formation by microorganisms, the presence of glucose and ethanol is mentioned. Sachin et al. [12] showed that the most durable biofilms were formed by strains grown under nutrient-poor conditions, and least intensively — in nutrient-rich medium (cerebral-heart broth). They found that minimal nutritional conditions can stimulate biofilm formation by *L. monocytogenes* [12]. Similar results were obtained by Zhou et al. [9] who showed that the recovery of *L. monocytogenes* from biofilm was the highest in minimal medium as compared to BHI. On the other hand, Pan et al. [13] showed that increase of sodium chloride concentration from 0.5% to 7.0% at 22.5°C and 30°C, stimulate *L. monocytogenes* strains to form the biofilm. The optimal concentration of sodium

chloride was 5.0% at 22.5°C. In addition, *L. monocytogenes* serotype 1/2a strains (more often isolated from food products) have been shown to form a biofilm with a higher density than serotype 4b strains, with higher glucose concentration and at higher temperature. These researchers also found that the addition of 3.0% and 5.0% of ethanol inhibited the formation of biofilms. The biofilm density of the tested strains increased with rising incubation temperature. Temperature, glucose and sodium chloride have a synergistic effect in the biofilm formation by *L. monocytogenes* strains [13]. Limited access to nutrients in minimal media can stimulate stress in *L. monocytogenes* cells, thereby inducing enhanced biofilm formation. Stress factors include temperature, limited availability of nutrients and increased oxygen availability [5]. In the present study, it was found that the highest cell recovery of *L. monocytogenes* from a biofilm with increasing addition of sheep blood was observed for the strain isolated from the human blood. However, Barbarosa et al. [14] who assessed the ability of biofilm formation by *L. monocytogenes* on the polystyrene under osmotic stress showed that clinical strains were less effective in producing biofilms compared to strains isolated from food [14].

Conclusions

The addition of sheep blood to the medium at concentrations up to 20.0% increases the number of cells recovered from the biofilm for all studied *L. monocytogenes* strains whereas medium with 50.0% blood stimulates the biofilm formation only by the strain isolated from blood. It seems clear that limited availability of nutrients stimulates *L. monocytogenes* to form a biofilm.

Conflict of interest: Authors declare no conflict of interest.

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References

- Schmid MW, Ng EYW, Lampidis R, et al. Evolutionary history of the genus *Listeria* and its virulence genes. *Syst Appl Microbiol*. 2005; 28(1): 1–18, doi: [10.1016/j.syapm.2004.09.005](https://doi.org/10.1016/j.syapm.2004.09.005), indexed in Pubmed: [15709360](https://pubmed.ncbi.nlm.nih.gov/15709360/).
- Jeyaletchumi P, Tunung R, Margaret SP, et al. Detection of *Listeria monocytogenes* in foods. *International Food Research Journal*. 2010; 17: 1–11.
- Gandhi M, Chikindas ML. *Listeria*: A foodborne pathogen that knows how to survive. *Int J Food Microbiol*. 2007; 113(1): 1–15, doi: [10.1016/j.ijfoodmicro.2006.07.008](https://doi.org/10.1016/j.ijfoodmicro.2006.07.008), indexed in Pubmed: [17010463](https://pubmed.ncbi.nlm.nih.gov/17010463/).
- Colagiorgi A, Di Ciccio P, Zanardi E, et al. A Look inside the *Listeria monocytogenes* Biofilms Extracellular Matrix. *Microorganisms*. 2016; 4(3), doi: [10.3390/microorganisms4030022](https://doi.org/10.3390/microorganisms4030022), indexed in Pubmed: [27681916](https://pubmed.ncbi.nlm.nih.gov/27681916/).
- Srey S, Jahid I, Ha SD. Biofilm formation in food industries: A food safety concern. *Food Control*. 2013; 31(2): 572–585, doi: [10.1016/j.foodcont.2012.12.001](https://doi.org/10.1016/j.foodcont.2012.12.001).
- Renier S, Hébraud M, Desvaux M. Molecular biology of surface colonization by *Listeria monocytogenes*: an additional facet of an opportunistic Gram-positive foodborne pathogen. *Environ Microbiol*. 2011; 13(4): 835–850, doi: [10.1111/j.1462-2920.2010.02378.x](https://doi.org/10.1111/j.1462-2920.2010.02378.x), indexed in Pubmed: [21087384](https://pubmed.ncbi.nlm.nih.gov/21087384/).
- Nowak J, Cruz CD, Palmer J, et al. Biofilm formation of the *L. monocytogenes* strain 15G01 is influenced by changes in environmental conditions. *J Microbiol Methods*. 2015; 119: 189–195, doi: [10.1016/j.mimet.2015.10.022](https://doi.org/10.1016/j.mimet.2015.10.022), indexed in Pubmed: [26524221](https://pubmed.ncbi.nlm.nih.gov/26524221/).
- da Silva EP, De Martinis EC. Current knowledge and perspectives on biofilm formation: the case of *Listeria monocytogenes*. *Appl Microbiol Biotechnol*. 2013; 97(3): 957–968, doi: [10.1007/s00253-012-4611-1](https://doi.org/10.1007/s00253-012-4611-1), indexed in Pubmed: [23233205](https://pubmed.ncbi.nlm.nih.gov/23233205/).
- Zhou Q, Feng X, Zhang Q, et al. Carbon catabolite control is important for *Listeria monocytogenes* biofilm formation in response to nutrient availability. *Curr Microbiol*. 2012; 65(1): 35–43, doi: [10.1007/s00284-012-0125-4](https://doi.org/10.1007/s00284-012-0125-4), indexed in Pubmed: [22526570](https://pubmed.ncbi.nlm.nih.gov/22526570/).
- Van Houdt R, Michiels CW. Biofilm formation and the food industry, a focus on the bacterial outer surface. *J Appl Microbiol*. 2010; 109(4): 1117–1131, doi: [10.1111/j.1365-2672.2010.04756.x](https://doi.org/10.1111/j.1365-2672.2010.04756.x), indexed in Pubmed: [20522145](https://pubmed.ncbi.nlm.nih.gov/20522145/).
- Xia FDI, Mallet A, Caliot E, et al. Capsular polysaccharide of Group B *Streptococcus* mediates biofilm formation in the presence of human plasma. *Microbes Infect*. 2015; 17(1): 71–76, doi: [10.1016/j.micinf.2014.10.007](https://doi.org/10.1016/j.micinf.2014.10.007), indexed in Pubmed: [25448634](https://pubmed.ncbi.nlm.nih.gov/25448634/).
- Kadam SR, den Besten HMW, van der Veen S, et al. Diversity assessment of *Listeria monocytogenes* biofilm formation: impact of growth condition, serotype and strain origin. *Int J Food Microbiol*. 2013; 165(3): 259–264, doi: [10.1016/j.ijfoodmicro.2013.05.025](https://doi.org/10.1016/j.ijfoodmicro.2013.05.025), indexed in Pubmed: [23800738](https://pubmed.ncbi.nlm.nih.gov/23800738/).
- Pan Y, Breidt F, Gorski L. Synergistic effects of sodium chloride, glucose, and temperature on biofilm formation by *Listeria monocytogenes* serotype 1/2a and 4b strains. *Appl Environ Microbiol*. 2010; 76(5): 1433–1441, doi: [10.1128/AEM.02185-09](https://doi.org/10.1128/AEM.02185-09), indexed in Pubmed: [20048067](https://pubmed.ncbi.nlm.nih.gov/20048067/).
- Barbosa J, Borges S, Camilo R, et al. Biofilm Formation among Clinical and Food Isolates of *Listeria monocytogenes*. *Int J Microbiol*. 2013; 2013: 524975, doi: [10.1155/2013/524975](https://doi.org/10.1155/2013/524975), indexed in Pubmed: [24489549](https://pubmed.ncbi.nlm.nih.gov/24489549/).