PREVALENCE AND BIOFILM FORMING ABILITY OF *LISTERIA MONOCYTOGENES* ISOLATED FROM MEAT

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Abstract: *Listeria monocytogenes* is a Gram-positive intracellular bacterium, which causes foodborne listeriosis. This organism can be introduced through many routes to food-processing environments and may become established on food-processing equipment. Examination of 130 meat samples was conducted and the *Listeria monocytogenes* prevalence was determined by using an ISO culture method and PCR method. The isolated strains' ability to form a biofilm was determined with the microplate (MP) method. Out of 130 meat samples examined, 22 (17%) contained *L. monocytogenes*. The majority (n=13; 59%) of the strains were characterized by a no biofilm producer. Four of the of *Listeria monocytogenes* strains (18,2%) showed strong and five of them (22,7%) moderate ability to form biofilm

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Introduction

Listeria monocytogenes is a Gram-positive, psychotropic foodborne pathogen. The aim of this study was to investigate the incidence of *Listeria monocytogenes* in raw meat. Moreover, the aim of the study was to determine the isolated strains' ability to form biofilm.

Literature review

Listeria monocytogenes is able to grow at different temperature (including refrigeration temperature) ranging from 2 to 52 °C, and in food having low pH value (4,4) or high salt concentration (14%). The minimum water activity (a_w) for the growth of *L. monocytogenes* in food is 0.92. The bacteria grow better (short generation and lag time) at the alkaline pH and high a_w . *Listeria monocytogenes* grows well in the presence or absence of oxygen. High concentrations of carbon dioxide are necessary to inhibit growth. At least 13 different serotypes of *L. monocytogenes* are known. Most cases are caused by serotypes 1/2a, 1/2b and 4b. The majority of reported foodborne outbreaks have been caused by serotype 4b (Ryser and Marth, 2007; Kramarenko et al., 2013). The minimum infective dose of *L. monocytogenes* is unknown, but is believed to vary in accordance with the immunity of the infected individual and the strain type (Bailey et al., 1989). Listeriosis arises from food contamination with *Listeria monocytogenes* causing encephalitis, septicaemia and miscarriage. The first epidemiologically confirmed foodborne outbreak of listeriosis occurred in 1981 in Canada and was linked to the consumption of Coleslaw salad. Other outbreaks of human listeriosis have been associated with milk, soft cheese, pate, jellied pork tongue, rillettes and other foods of animal or vegetable origin (Capita et al. 2001). Some *L. monocytogenes* strains attach to environmental surfaces and form biofilms, perhaps by using peritrichous flagella encoded by *flaA* gene. Microorganisms growing in biofilms are protected against cleaning and disinfection and are difficult to eradicate. *L. monocytogenes* can attach and grow on abiotic surfaces including all types of foods processing surface such as glass, stainless steel, polypropylene, and rubber after contact times as short as 20 minutes. The biofilm formation renders the organisms more resistant to harsh environmental conditions, such as food processing, antibiotics, and detergents (Fux et al. 2005; Mafu et al., 1990).

Materials and Methods

Isolation of *Listeria* strains

A total of 130 samples were obtained from several local markets in Olsztyn, north-west Poland. Samples consisted of poultry meat: duck, chicken, turkey (n=40), beef meat (n=45), and pork meat (n=45). All the samples were kept at 4°C during transport and during storage before analysis. The analysis was done within 4 hours of purchase.

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Culture method of *Listeria monocytogenes* isolation was based on the International Standard ISO 11290-1:1996. For primary enrichment, 25 g of each of the collected food samples was transferred to 225 ml of Half-Fraser broth (Merck, Poland), homogenized using a stomacher, next incubated overnight at 37°C. After 24 h incubation of the primary enrichment broth, 1 ml of the culture obtained was transferred to 10 ml of secondary enrichment broth, the Fraser broth (Merck, Poland). Inoculated Fraser broth was incubated for 48 h at 37°C. After incubation, a portion of the culture was taken with a loop for inoculating of the surface of Oxford and Ottawiani and Agosti agars (Merck, Poland). After incubation for 48 h at 37°C the plates were examined for the presence of colonies presumed to be *Listeria* spp. Colonies suspected to be *Listeria* spp. were selected for further confirmatory tests indicated in ISO 11290-1:1996. Finally, the colonies identified as *L. monocytogenes* were confirmed by PCR. Prior to analysis, isolates were stored in a Microbank at -80°C (Biocorp, Poland).

PCR identification of *Listeria monocytogenes*

Identification using a Multiplex PCR was performed with primers and conditions described previously (Szymczak et al., 2011), specific for the *Listeria* sp. and *Listeria monocytogenes* based on the amplification of fragments of 16S rRNA gene sequence (938 bp) and *iap* (287 bp) (Table 1). In all PCR reactions used, amplified products were analyzed by electrophoresis through a 1,5% high resolution agarose gel (Promega) in 1 x TBE buffer pH 8,3. The sizes of the amplification products were estimated by comparison with a 100-bp molecular size ladder (Thermo Scientific, Fermentas). Gels were stained with ethidium bromide (Sigma-Aldrich) and visualized using the system for the documentation of fluorescently stained gels G-BOX F3 (Syngene) and analyzed using the program Gene Tools (Syngene). Each profile was visually compared with those obtained from the reference strains: *Listeria monocytogenes* NCIMB 13726.

Table 1: Primers used for the amplification of the *Listeria* spp. and *Listeria monocytogenes*

Table 1: Primers used for the amplification of the <i>Listeria spp.</i> and <i>Listeria monocytogenes</i>				
Primer	Sequence (5'-3')	Destination/target gene	Product size	References
U1	CAG CMG CCG CGG TAA TWC	<i>Listeria spp</i>	938 bp	Szymczak et al. 2011
LI1	CTC CAT AAA GGT GAC CCT			
Iap1	CGA ATC TAA CGG CTG GCA CA	<i>L. monocytogenes</i>	287 bp	
Iap2	GCC CAA ATA GTG TCA CCG CT			
Source: Authors				

Detection of biofilm formation by the microplate (MP) method

The method for assessment of biofilm formation by the MP method was based on the techniques proposed previously by Stepanovic et al. (2004) with some modifications described previously (Chajęcka-Wierzchowska, Zadernowska & Łaniewska-Trokenheim, 2016). Wells of a sterile 96-well flat-bottomed sterile polystyrene microtiter plates were filled with 200 µl of fresh sterile broth BHI (Merck, Poland). An overnight cultures (20 µl) of each strain with a cell density of 1×10^9 cells/ml were added in triplicate, onto the 96-well plates. Negative control wells contained broth only. The plates were covered and incubated aerobically at 36°C for 24 hours. The bacterial suspension was aspirated and each well was washed three times with 250 µL of PBS buffer (Sigma). After that, the biofilm was fixed with 200 µL of ethanol (99%) for 15 minutes, and later removed. The plates were dried at room temperature, stained with 200 µL of crystal violet solution used for Gram staining (Merck, Germany) for 5 minutes, washed in running water until the unbound crystal violet was removed and dried at room temperature. The dye bound to the adherent cells was re-solubilized with 160 µL of 33% (v/v) glacial acetic acid (Sigma) per well. Absorbance was read using an Infinite M1000 PRO plate reader (Tecan) at 570 nm. The optical density (ODs) of each strain was obtained by the arithmetic mean of the absorbance of three wells and this value was compared with the cut-off OD (OD_c) which was defined as three standard deviations above the mean OD of the negative control. The following classification was used for the determination of biofilm formation: no biofilm production ($OD \leq OD_c$), weak biofilm production ($OD_c < OD \leq 2 \times OD_c$), moderate biofilm production ($2 \times OD_c < OD \leq 4 \times OD_c$) and strong biofilm production ($4 \times OD_c < OD$).

Results and discussion

A total of 130 meat samples, including poultry, beef and pork meat were analysed for *Listeria monocytogenes*. Results are summarized in Table 2. The prevalence of *L. monocytogenes* in raw meats marketed in Olsztyn (Poland) was 17%. The percent occurrence of *Listeria monocytogenes* in the tested meat in descending order was poultry (20%), beef (9,8%) and pork (4,5%). Capita et al. (2001) isolated *Listeria monocytogenes* from 15% of poultry carcasses. A study conducted by Vitas (2004) in Navarra (Spain) revealed the presence of *Listeria monocytogenes* in 34.9% in minced pork and beef and 36.1% in poultry meat. No documented outbreaks of listeriosis have yet been directly attributed to the consumption of poultry meat. Nevertheless, the high prevalence of *L. monocytogenes* in poultry meat presents a potential risk, which may lead to outbreaks of listeriosis (Capita et al. 2001).

Table 2: Occurrence and biofilm forming ability of *Listeria monocytogenes* strains isolated from meat

Samples tested	Number of samples	<i>Listeria monocytogenes</i>			
		Number (%) of strains	Biofilm formation category		
			S	M	NBP
Poultry meat	40	8 (20)	2	2	4
Beef meat	45	4 (9,8)	0	2	2
Pork meat	45	10 (4,5)	2	1	7
Total	130	22 (17)	4	5	13

Abbreviations: S – strong, M – moderate, NBP – no biofilm producer

Source: Authors

The pathogens' ability to form biofilms has a twofold meaning. From a medical point of view, the ability to form it is a feature which proves the strain's virulence. Components of a biofilm matrix protect bacteria against an immune attack and against penetration of chemotherapeutics. However, in terms of their industrial significance, strains which can form biofilms of manufacturing surfaces can pose a big problem; they are more difficult to remove from those surfaces, they can be less sensitive to disinfectants and they often cause secondary (cross) infections in the food industry (Shi & Zhu X 2009, Zadernowska, Chajęcka-Wierzchowska & Łaniewska-Trokenheim 2017). Strains of *L. monocytogenes* are recurrently found on surfaces in the food industry, notably in refrigerated premises, even though these are routinely cleaned and disinfected (Carpentier et al. 2004). The majority of the *Listeria monocytogenes* strains were characterized by a no biofilm producer (n=13), or moderate ability to form a biofilm (n=5), only four of the investigate strains showed a strong ability to produce biofilm (Table 2). In a study conducted by Barbosa et al. (2013) revealed that most food isolates were classified as weak or moderate biofilm formers, although the majority were weak. In a study conducted by Kalmkoff et al. (2001), significant differences were found in the ability of various *L. monocytogenes* strains to attach to a test surface. In monoculture, the majority of strains did not form biofilms which coincides with our results.

Conclusions

Our results and data presented by other authors demonstrate the ubiquitous occurrence of *L. monocytogenes* in raw chicken, beef and pork meat. Since *L. monocytogenes* may be present in meat the consumption of raw or undercooked meat can be an important factor in the transmission and epidemiology of *Listeria monocytogenes* infection. It is also necessary to raise consumer awareness regarding secondary infections, which can occur during the transport of meat from a shop or during its processing. Regular monitoring of *Listeria monocytogenes* prevalence in food are necessary for disease surveillance and tracing the epidemic outbreaks.

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