

Characteristics of *Listeria monocytogenes* Strains Isolated from Milk and Humans and the Possibility of Milk-Borne Strains Transmission

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Abstract

Listeria monocytogenes is the etiological factor of listeriosis. The main source of these organisms is food, including dairy products. The aim was to determine the multiple correlations between the drug susceptibility, virulence genes (VGs), and biofilm formation on silicone teat cups of milk-borne and human *L. monocytogenes* strains. The spread of *L. monocytogenes* via contaminated teat rubbers was assessed. The *L. monocytogenes* strains recovered from milk (18), human blood (10), and the reference strain ATCC®19111™ were used in the study. Penicillin resistance was the most prevalent resistance in the milk isolates (n = 8; 44.4%), whereas among clinical strains erythromycin resistance was predominating – (n = 6; 60%). The most frequent VGs among strains isolated from milk were *hlyA* (100%) and *plcB* (100%) whereas in strains isolated from blood – *hlyA* (100%) and *prfA* (90%). All tested VGs were present in 50% of blood isolates and 11% of milk-borne strains. The strains isolated from milk formed a significantly stronger biofilm. The strains with more numerous virulence genes were resistant to more antibiotics and formed a stronger biofilm. It was shown that contaminated teat cups might contribute to the transmission of *L. monocytogenes* in the herd. It seems reasonable to monitor the occurrence of *L. monocytogenes* biofilm in a dairy processing environment.

Key words: *Listeria monocytogenes*, biofilm formation, drug susceptibility, bacteria transmission, virulence genes frequency

Introduction

Listeria monocytogenes is Gram-positive, facultatively anaerobic, non-spore forming bacteria. *L. monocytogenes* causes listeriosis and is one of the most dangerous food-borne zoonotic pathogens (Schlech and Acheson 2000). Listeriosis mainly occurs in immunocompromised people, neonates, pregnant women, the elderly, and AIDS patients (Lawley et al. 2008; Jamali et al. 2013; Doijad et al. 2015). The major source of human infection is food contaminated with the pathogen, including raw milk and milk products. A recent

trend to purchase fresh, unprocessed products from local suppliers has contributed to an increase in the consumption of unpasteurized milk and products thereof (Seremak-Bulge et al. 2013). Such products may be the source of pathogenic bacteria and lead to serious infections (Jamali and Radmehr 2013; Gould et al. 2014).

According to the European Food Safety Authority (EFSA) report in 2017 all tested milk samples included in the RTE (ready-to-eat) products category were in line with Food Safety Criteria (FSC). One of 85 samples (1.2%) from two member countries, in the category “Raw cow’s milk for direct consumption” in the

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retail trade, was positive for *L. monocytogenes* (EFSA 2018). In Poland and other European countries, the number of infections caused by this microorganism is constantly increasing. In 2017 2480 cases of listeriosis were recorded in the EU countries, while in 2013 only 1763 cases were found. The incidence rate per 100 000 inhabitants was 0.26 and 0.32, respectively, in 2016 and 2017 (EFSA 2018). In 2018 124 cases of listeriosis were reported in Poland that is a 20% increase when compared to 2015 (NIZP-PZH 2019). Listeriosis associated with raw milk consumption was reported in 2014 in two patients from California and Florida. The most likely source of *Listeria* spp. was raw chocolate milk (CDC 2016). In Los Angeles County (California) during an outbreak associated with the consumption of the contaminated cheese, the death of 48 out of 142 patients was recorded (Linnan et al. 1988). Another listeriosis outbreaks linked to the contaminated cheese took place in Germany in 2006–2007 (Koch et al. 2010), and in Spain in 2008–2009 (Jackson et al. 2011) where 189 and 8 cases were reported, respectively.

Despite the improvement in the hygiene of the production process, *L. monocytogenes* poses still a serious problem in the food processing plants, including dairies. Such environment favors biofilm formation and bacterial survival (Latorre et al. 2010). Since *L. monocytogenes* multiplies easily and quickly on improperly cleaned dairy appliances, biofilm formation starts within 20 minutes after bacterial contact with the surface (Hayes et al. 1986; Weiler et al. 2013). In dairy plants, *L. monocytogenes* colonizes processing surfaces, floors, equipment, and niches that are difficult to clean (e.g. hard to reach cavities), and becomes the potential source of milk and milk product contamination (Unnerstad et al. 1996; Latorre et al. 2010). Bacteria forming biofilm survive longer and are more resistant to disinfectants and mechanical washing (Frank and Koffi 1990; Walker et al. 1990; Meyer-Broseta et al. 2003). Therefore, it is of great importance to prevent biofilm formation on milking equipment to reduce the risk of milk contamination and, in consequence, human infections (Latorre et al. 2010).

Although most *L. monocytogenes* strains are susceptible to antimicrobial agents used in human and animal medicine, multidrug-resistant strains are increasingly frequently isolated. This is mainly due to the overuse of antibiotics in animal husbandry (Jamali et al. 2013). Also, several virulence factors enable *L. monocytogenes* to infect and spread in the host (Park et al. 2012). *L. monocytogenes* possesses many virulence genes responsible for the invasion of host cells (*inlA*, *inlB*, and *iap*), phagosomal escape (*hlyA*, *plcA*, and *plcB*) and cell to cell spread (*actA*) (Hamon et al. 2006).

This study aimed was to determine the multiple correlations between drug susceptibility, virulence genes

and biofilm formation on silicone teat cups from milking machines of milk-borne and human *L. monocytogenes* strains. The spread of *L. monocytogenes* through contaminated teat rubbers was also assessed.

Experimental

Materials and Methods

Materials. Milk samples, obtained in Poland from cows without clinical signs of *mastitis*, were collected in 2015. For each cow, a sample of 100 ml from all the four teats was collected to one sterile container. Of 380 milk samples, 21 (5.5%) were positive for *L. monocytogenes*. Ten genetically different strains (a genetic similarity previously determined for the diagnostic reasons) isolated from the blood of patients by dr. A. Jurasz (University Hospital No. 1 in Bydgoszcz, Poland) were used to evaluate their drug susceptibility and ability to form biofilms. There was no epidemiological link between milk and blood isolate groups. The reference strain *L. monocytogenes* ATCC® 19111™ was included in the study. This strain is widely used as the reference strain in many studies, including biofilm formation assessment.

Isolation of *L. monocytogenes* from milk. Analysis of the intermediate and finished product samples was carried out following the ISO 11290-1 (ISO 2017). To isolate *L. monocytogenes*, 25 ml of milk was added to 225 ml of half-Fraser broth (Merck, Poland) and incubated for 24 h at 30°C. Then 0.1 ml of the culture was transferred into 10 ml of Fraser broth (Merck, Poland) and the secondary selective enrichment was performed at 37°C for 48 h. Finally, the culture was plated onto ALOA agar (ChromoCult *Listeria* Selective Agar®, Merck, Poland), and OXFORD Agar (Oxoid, United Kingdom), and incubated for 24 h at 37°C.

Identification of the strains isolated from milk. Initial species identification was performed based on morphological traits on the ALOA agar (Merck, Poland). The typical colony of *L. monocytogenes* is a turquoise and blue one surrounded with a turbidity zone.

Then, multiplex PCR was performed. For the genus *Listeria* identification primers (L1, L2) (Oligo.pl, Poland) based on the 16S rRNA sequence were used (Border et al. 1990) whereas primers (LM1, LM2) based on the sequence of the *hly* gene allowed the species identification (Bansal 1996).

DNA was isolated using the Genomic Mini AX Bacteria Spin kit (A&A Biotechnology, Poland), according to the manufacturer's instructions. Amplification was performed in the mixture of 25 µl, containing: 1 × PCR buffer (Promega, United States), 2.0 mM MgCl₂ (ABO, Poland), 1.25 mmol dNTPs (Promega, United States),

Table I
Primers used for identification of *L. monocytogenes*.

Primer	Primer sequence (5' – 3')	Size of PCR product (bp)	Amplification conditions
L1	CAG CAG CCG CGG TAA TAC	938	Initial denaturation – 94°C/2 min 30 cycles: denaturation – 94°C/30 s annealing, – 50°C/30 s elongation – 72°C/1 min final elongation – 72°C/5 min
L2	CTC CAT AAA GGT GAC CCT		
LM1	CCT AAG ACG CCA ATC GAA	750	
LM2	AAG CAC TTG CAA CTG CTC		

0.5 µM of each of the primers (Oligo.pl, Poland), 1.0 U Taq DNA polymerase (Promega, United States), ultrapure water (Merck, Poland) and 2.0 µl DNA. The amplification conditions and primer sequences are presented in Table I. *L. monocytogenes* strain ATCC 19111 was used as the reference strain. PCR products were separated on 1.5% agarose gel for 75 min at 80V and stained with a Midori Green dye (NIPPON Genetics EUROPE GmbH, Germany).

Determination of genetic relatedness of *L. monocytogenes* strains isolated from milk. All *L. monocytogenes* isolates were genotyped using Random Amplification of Polymorphic DNA (RAPD) with the nonspecific primer OPA-11 (5'-CA AT CG CC GT-3') (Ozbey et al. 2006). Reactions were carried out in a mixture of 25 µl, containing: 1 × PCR buffer with 2.0 mM MgCl₂ (Promega, United States), 1.5 mM MgCl₂ (ABO, Poland), 200 µM dNTPs (Promega, United States), 1.0 µM primer OPA-11 (Oligo.pl, Poland), 1.25 U Taq DNA polymerase (Promega, United States), ultrapure water (Merck, Poland), and 3.0 µl DNA. The reaction consisted of six cycles of initial stage: denaturation (94°C/1 min), annealing (30°C/2 min) and elongation (72°C/1 min), followed by 35 cycles of: denaturation (94°C/15 s), annealing (37°C/1 min) and elongation (72°C/45 s), and the final elongation (72°C/10 min). PCR products were separated on 1.5% agarose gel for 150 min at a voltage of 80 V, and stained with a Midori Green dye (NIPPON Genetics EUROPE GmbH, Germany).

To determine the degree of genetic relationship between the isolates, the phylogenetic dendrogram was drawn in the program Phoretix 1D Pro (TotalLab). Data clustering was performed using the UPGMA hierarchic grouping technique with the Dice coefficient.

The frequency of the genes encoding virulence factors in milk and blood isolates. *L. monocytogenes* strains from milk (18) and blood (10) were examined for the multiplex PCR reactions. The study included the following virulence genes: *actA* (actin assembly-inducing protein), *hlyA* (listeriolysin O), *iap* (extracellular protein p60), *inlA* (internalin A), *inlB* (internalin B), *plcA* (phosphatidylinositol-specific phospholipase C), *plcB* (phosphatidylcholine-specific phospholipase C), and *prfA* (positive regulatory factor PrfA).

Two multiplex-PCR reactions were optimized to detect these virulence genes. The first included the detection of *iap*, *hlyA*, *inlB*, and *plcB*, and the second one – *actA*, *inlA*, *plcA*, and *prfA* genes. The reaction mixture (25 µl) contained: 1 × PCR buffer with 2.0 mM MgCl₂ (Promega, United States), 6.0 mM MgCl₂ (ABO, Poland), 1.0 mM dNTPs (Promega, United States), 1.0 µM of each primer (Oligo.pl, Poland) (Table II), 3.0 U Taq DNA polymerase (Promega, United States), ultrapure water, and 3.0 µl DNA. The course of PCR was as follows: the initial denaturation (95°C/2 min), 35 cycles of denaturation (95°C/15 s), annealing (60°C/30 s) and elongation (72°C/1.5 min) and the final elongation (72°C/10 min). The *L. monocytogenes* strain IW41 was used as the reference strain. PCR products were separated on 1.5% agarose gel and stained with a Midori Green dye (NIPPON Genetics EUROPE GmbH, Germany). The Perfect 100 bp DNA Ladder (EurX, Poland) was used.

Evaluation of drug susceptibility of *L. monocytogenes* strains. Drug susceptibility of 18 milk and ten blood isolates was determined using the disk diffusion method on the Mueller-Hinton agar with 5% defibrinated horse blood and β-NAD at a concentration of 20 mg/l (MH-F, bioMérieux, France). For each strain, a suspension of 0.5 McFarland's scale (7.6 × 10⁷ CFU/ml ± 9.4 × 10⁶ CFU/ml) in sterile saline was prepared. The disks with penicillin (1 U), ampicillin (2 µg), meropenem (10 µg), erythromycin (15 µg) and cotrimoxazole (1.25–23.75 µg) (Becton Dickinson, United States) were used. Incubation of antibiograms was conducted in the atmosphere enriched in 5% CO₂ at 35°C for 18 h. The results were interpreted according to the recommendations of EUCAST v. 8.0.

Evaluation of the biofilm formation ability of *L. monocytogenes* strains. Quantitative evaluation of biofilm formation by *L. monocytogenes* strains was performed on irradiated fragments of silicone teat cups from milking machines. The fragments of 1 × 1 cm were used in the study.

The intensity of biofilm formation was determined using a quantitative method by Kwiecińska-Piróg et al. (2011) with some modifications. The study was conducted on one clinical strain, one strain isolated from milk, and the reference strain ATCC 19111. The

Table II
Primers used for the detection of virulence genes in *Listeria monocytogenes* strains
(Franciosa et al. 2005; Rawool et al. 2007).

Primer	Primer sequence (5' – 3')	Size of PCR product (bp)
<i>actA</i> – F	CGC CGC GGA AAT TAA AAA AAG A	839
<i>actA</i> – R	ACG AAG GAA CCG GGC TGC TAG	
<i>hlyA</i> – F	GCA GTT GCA AGC GCT TGG AGT GAA	456
<i>hlyA</i> – R	GCA ACG TAT CCT CCA GAG TGA TCG	
<i>iap</i> – F	ACA AGC TGC ACC TGT TGC AG	131
<i>iap</i> – R	TGA CAG CGT GTG TAG TAG CA	
<i>inlA</i> – F	CAG GCA GCT ACA ATT ACA CA	2 341
<i>inlA</i> – R	ATA TAG TCC GAA AAC CAC ATC T	
<i>inlB</i> – F	AGG AGA GGA TAG TGT GAA	1 905
<i>inlB</i> – R	TTA TTT CTG TGC CCT TAA	
<i>plcA</i> – F	CTG CTT GAG CGT TCA TGT CTC ATC CCC C	1 484
<i>plcA</i> – R	ATG GGT TTC ACT CTC CTT CTA C	
<i>plcB</i> – F	GCA AGT GTT CTA GTC TTT CCG G	794
<i>plcB</i> – R	ACC TGC CAA AGT TTG CTG TGA	
<i>prfA</i> – F	CAT GAA CGC TCA AGC AGA AG	706
<i>prfA</i> – R	AAT TTT CCC AAG TAG CAG GA	

sterile fragments of rubber (3 replications for each strain) were placed in tubes containing 3 ml of the bacterial suspensions in the Brain Heart Infusion Broth (BHI) (Merck, Poland) (0.5 of the McFarland scale). Incubation was conducted in the aerobic atmosphere at 37°C for 72 h, and the medium was replaced with a sterile one every 24 h. At each exchange of the medium, the rubber fragments were rinsed with sterile PBS (Phosphate Buffered Saline) (BTL, Poland). The fragments of rubbers incubated in the sterile BHI medium (Merck, Poland) were used as negative controls. After incubation, the samples were rinsed with PBS, placed in a tube containing 3 ml of PBS, and sonicated for 10 minutes (30 kHz, 150W) with the sonicator Ultrasonic DU-4 (Nickel-Electro Ltd.). Then, the samples were shaken for 10 minutes (400 rpm), and the serial 10-fold dilutions were prepared and inoculated on the Columbia Agar medium with 5% sheep blood (Becton Dickinson, United States). After 24-hours incubation at 37°C, the number of colonies per 1 cm² of the fragment's surface (CFU/cm²) was calculated.

The intensity of biofilm formation by *L. monocytogenes* was observed under the confocal microscope. For this purpose, very thin rubber slides were prepared and the biofilms were grown, as described above. The biofilm-forming cells on the rubber slides were then stained with the LIVE/DEAD BacLight Bacterial Viability Kit (ThermoFisher Scientific, United States), according to the manufacturer's instructions.

The spread of *L. monocytogenes* through contaminated teat rubbers. *Transmission of L. monocytogenes*

on the skin of the udder and into the milk. The radiant sterilized, purified from fat, pieces of cow's udder skin (1 × 1 cm) were used in the experiment. Biofilm formation on sterile teat rubbers was assessed as described above (section "Evaluation of biofilm formation ability of *L. monocytogenes* strains"). Two of the strongest and weakest biofilm-forming strains of *L. monocytogenes* derived from both milk and blood as well as the reference strain were selected for the study.

To evaluate the transmission of bacteria from the biofilm formed on the teat rubbers to the udder skin (according to our method), a piece of skin was rubbed with the contaminated rubber in two directions. This simulated the insertion and removal of the teat into the milking cup. For each strain, eight skin fragments were used in six replicates. After swabbing, each piece of skin (1–3 replicates) was placed in sterile PBS (BTL, Poland) and subjected to a 10-minute sonication. Subsequently, the serial 10-fold dilutions were made and plated onto Columbia Agar with 5% sheep blood (Becton Dickinson, United States). After 24 hours at 37°C, the number of bacteria in 1 cm² of the udder skin was calculated. Also, to test the proliferation of *L. monocytogenes* on the skin, fragments (repeats 4–6) were left at 25°C for 12 hours. Subsequently, the samples were placed in sterile PBS, sonicated and plated onto Columbia Agar with 5% sheep blood (Becton Dickinson, United States).

To evaluate the transmission of *L. monocytogenes* from the biofilm formed on the teat rubbers to milk, each rubber was flushed with 100 ml of UHT milk. Then, 0.1 ml of milk was plated onto Columbia Agar

medium with 5% sheep blood (Becton Dickinson, United States), incubated at 37°C for 24 hours, and the number of bacteria in 1 ml of milk was determined.

Transmission of L. monocytogenes in udder – teat rubber – udder model. Two of the strongest and weakest biofilm-forming strains of *L. monocytogenes* derived from milk and blood and the reference strain were selected for the study.

The bacterial suspensions in sterile PBS (BTL, Poland) (0.5 McF) were prepared and 50 µl of the suspension (six replicates) was poured on the sterile skin fragments. After drying at room temperature, the sterile teat rubber piece was rubbed in two directions with the contaminated skin. Then, the rubber pieces were placed (repeats 1–3) in sterile PBS (BTL, Poland) and sonicated. The number of bacteria per 1 cm² of rubber was determined by plating the sample into Columbia Agar with 5% sheep blood (Becton Dickinson, United States), and incubation at 37°C for 24 hours. The remaining contaminated teat rubber fragments (repeats 4–6) were used to rub six sterile fragments of the udder skin. The number of bacteria transferred to them as well as proliferation on the udder skin was determined as described previously (“*Transmission of L. monocytogenes on the skin of the udder and into the milk*”).

Statistical analysis. The statistical analysis of the results was performed using the software Statistica 12 PL (StatSoft).

The frequency of the genes encoding virulence factors in *L. monocytogenes* strains isolated from milk and blood was established. Statistical analysis of the results

was carried out using the chi-square test and a Fisher exact test, at the significance level $\alpha=0.05$. The virulence profiles of the strains tested from both groups were also determined.

The number of bacteria re-isolated from biofilm was averaged separately for both groups of strains and compared with each other and with the reference strain using the analysis of variance ANOVA and the post hoc Bonferroni test, at the significance level $\alpha=0.05$.

The multiple correlations between antibiotic resistance, virulence genes, and intensity of biofilm formation among clinical strains and milk isolates were tested. Single correlations between antibiotic resistance and prevalence of virulence genes, antibiotic resistance and intensity of biofilm formation, and incidence of virulence genes and the intensity biofilm formation were also evaluated. Correlation coefficients were evaluated according to Guilford’s scale.

Results

Of the 380 milk samples tested, 21 (5.5%) were positive for *L. monocytogenes*. Since four isolates were genetically identical, finally 18 unrelated genetically strains were subjected to evaluation of drug susceptibility and the ability to form a biofilm (Fig. 1).

Assessment of the occurrence of virulence genes. The number of virulence genes varied in *L. monocytogenes* strains (Fig. 2). The most prevalent, found in all tested strains, was the *hlyA* gene. The *plcB* gene was

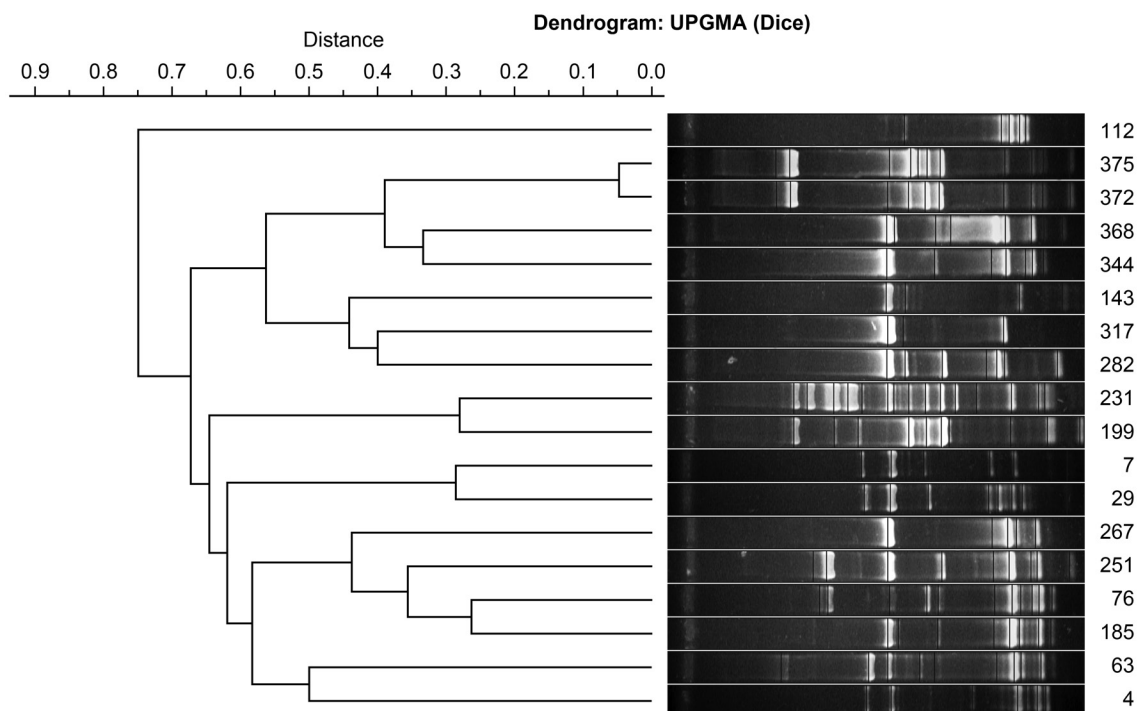


Fig. 1. Genetic similarity of tested *Listeria monocytogenes* strains.

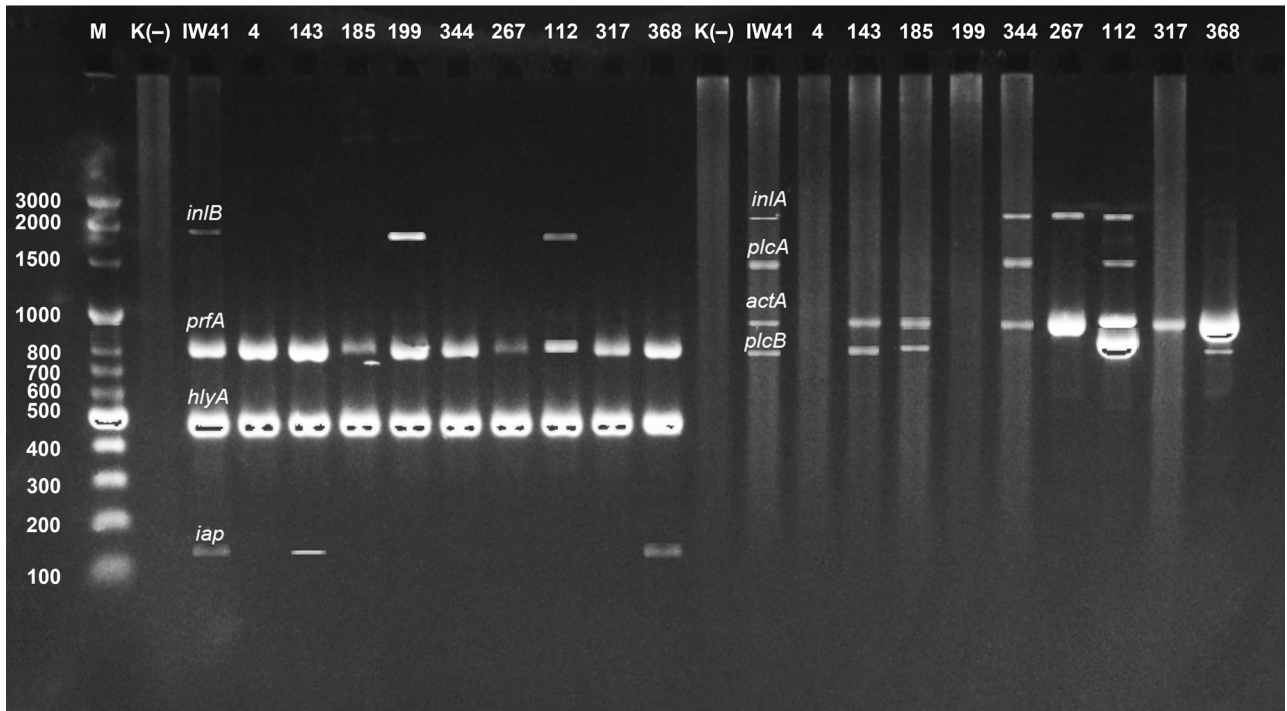


Fig. 2. Electrophoregram presenting the occurrence of virulence genes among the strains tested (M – marker, K+ – positive control (IW41), line 4, 143, 185, 199, 344, 267, 112, 317, 368 – *L. monocytogenes* strains from milk, K (-) – negative control).

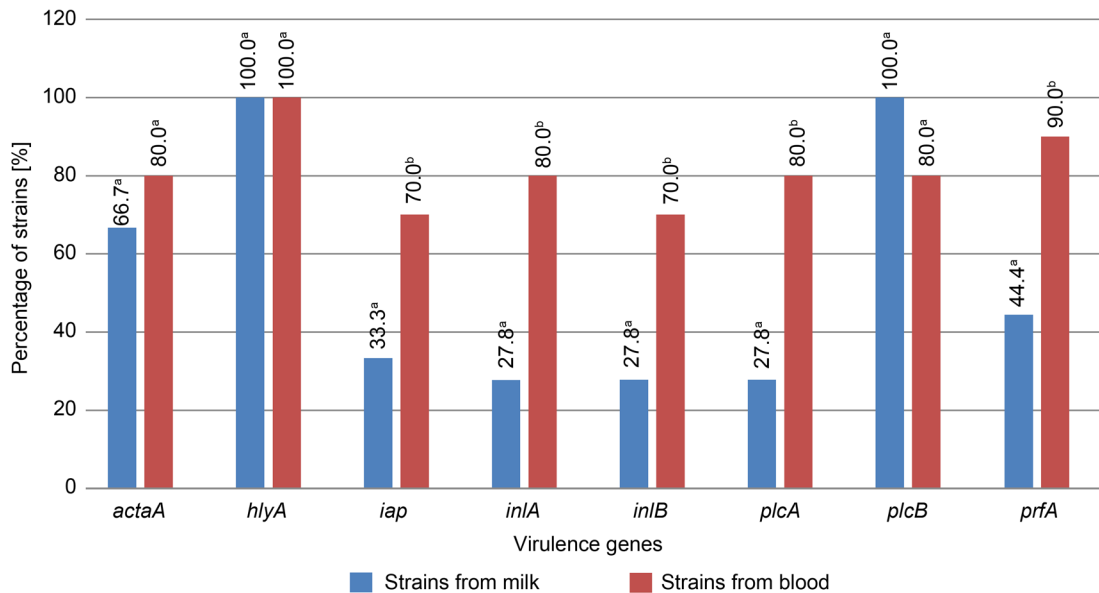


Fig. 3. Prevalence of virulence genes among *L. monocytogenes* strains isolated from milk and blood.

found in all milk isolates, whereas the *prfA* gene was detected in 90.0% of blood strains (Fig. 3). The occurrence of the remaining genes ranged from 27.8% to 80% of the strains tested. The reference strain of *L. monocytogenes* ATCC 19111 had all virulence genes investigated. The occurrence of the genes *iap*, *inlA*, *inlB*, *plcA*, and *prfA* was significantly higher in the strains isolated from the blood than in milk strains ($p \leq 0.05$).

Among *L. monocytogenes* strains isolated from milk, 11 different profiles of virulence genes were detected.

None of the profiles was represented by more than 2 strains (Table III). Six (33.3%) of *L. monocytogenes* strains from milk possessed three virulence genes. Two strains with all virulence genes investigated were identified. Among the *L. monocytogenes* isolates from the human blood six gene profiles were identified. The most frequent profile included all virulence genes tested (five strains, 50.0%) (Table III).

Evaluation of drug susceptibility of the strains. It was found that strains derived from milk were the

Table III
The profiles of virulence genes in strains of *L. monocytogenes* isolated from milk and from the blood.

Profile	Virulence genes	Number of strains	The strain identification number
Strains isolated from milk			
I	<i>hlyA, plcB</i>	2	4, 63
II	<i>hlyA, iap, plcB</i>	2	7, 29
III	<i>hlyA, inlB, plcB</i>	2	199, 231
IV	<i>actA, hlyA, plcB</i>	2	282, 317
V	<i>actA, hlyA, plcB, prfA</i>	2	76, 185
VI	<i>actA, hlyA, iap, plcB, prfA</i>	2	143, 368
VII	<i>actA, hlyA, iap, inlA, inlB, plcA, plcB, prfA</i>	2	372, 375
VIII	<i>actA, hlyA, inlA, plcB</i>	1	267
IX	<i>actA, hlyA, inlA, plcA, plcB</i>	1	344
X	<i>actA, hlyA, plcA, plcB, prfA</i>	1	251
XI	<i>actA, hlyA, inlA, inlB, plcA, plcB, prfA</i>	1	112
Strains isolated from the blood			
I	<i>actA, hlyA, iap, inlA, inlB, plcA, plcB, prfA</i>	5	1, 2, 5, 6, 9
II	<i>actA, hlyA, iap</i>	1	4
III	<i>actA, hlyA, iap, prfA</i>	1	10
IV	<i>hlyA, inlA, plcA, plcB, prfA</i>	1	3
V	<i>hlyA, inlA, inlB, plcA, plcB, prfA</i>	1	8
VI	<i>actA, hlyA, inlA, inlB, plcA, plcB, prfA</i>	1	7

most frequently resistant to penicillin (8; 44.4%) and erythromycin (7; 38.9%). In turn, among strains isolated from blood, four (4, 40.0%) were resistant only to erythromycin, and another two strains (2, 20.0%) were

resistant to erythromycin and meropenem (Table IV). Amongst all strains studied eight different profiles of drug susceptibility were found. Profile A was represented by 12 (42.86%) isolates susceptible to all the

Table IV
The profiles of drug resistance/susceptibility of strains of *L. monocytogenes* isolated from milk and the blood.

Profile	Drug resistance/ susceptibility	Number of strains		Total (n = 28)
		Strains from milk (n = 18) (the strain identification number)	Strains from the blood (n = 10) (the strain identification number)	
A	R: – S: P, AM, MEM, E, SXT	8 (44.44%) (7, 29, 63, 199, 231, 282, 317)	4 (40.0%) (3, 4, 8, 10)	12 (42.86%)
B	R: P S: AM, MEM, E, SXT	3 (16.67%) (76, 185, 251)	0 (0.0%)	3 (10.71%)
C	R: E S: P, AM, MEM, SXT	1 (5.56%) (267)	4 (40.0%) (1, 2, 7, 9)	5 (17.86%)
D	R: P, E S: AM, MEM, SXT	1 (5.56%) (143)	0 (0.0%)	1 (3.57%)
E	R: E, SXT S: P, AM, MEM	1 (5.56%) (344)	0 (0.0%)	1 (3.57%)
F	R: P, AM, MEM, SXT S: E	1 (5.56%) (368)	0 (0.0%)	1 (3.57%)
G	R: P, AM, MEM, E, SXT S: – – –	3 (16.67%) (112, 372, 375)	0 (0.0%)	3 (10.71%)
H	R: MEM, E S: P, AM, SXT	0 (0.0%)	2 (20.0%) (5, 6)	2 (7.14%)

R – resistant

S – susceptible

P – penicillin, AM – ampicillin, MEM – meropenem, E – erythromycin, SXT – cotrimoxazole

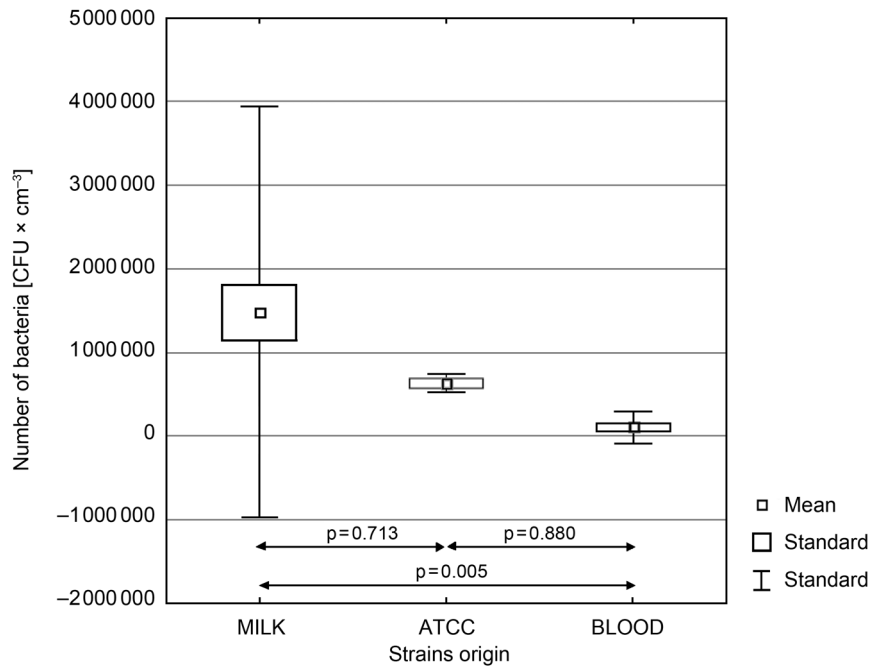


Fig. 4. Differences in biofilm formation among clinical *L. monocytogenes* strains and the strains isolated from milk.

antibiotics tested, eight isolates (44.4%) from milk and four (40.0%) from the blood. We found three (16.7%) strains that were resistant only to penicillin (profile B), and five (17.86%) – resistant only to erythromycin (profile C). At the same time, two (11.1%) strains were resistant to two antibiotics; one of them to penicillin and erythromycin (profile D) and the other to erythromycin and cotrimoxazole (profile E). Further, one strain (5.6%) isolated from milk was susceptible only to erythromycin (profile F), and three (16.7%) strains isolated from milk were resistant to all antibiotics tested (profile G) (Table IV). In turn, among clinical strains of *L. monocytogenes*, the most prevalent was resistance to erythromycin (60.0%). We found also five isolates resistant only to erythromycin (profile C), one of them was acquired from milk and four were isolated from blood. Further, two (20.0%) strains isolated from blood were resistant to meropenem and erythromycin (profile H) (Table IV).

Evaluation of ability to form biofilm by *L. monocytogenes* strains. It was found that isolates derived from milk formed a biofilm with significantly higher efficiency than the clinical isolates from the blood ($p=0.005$) (Fig. 4). The number of the cells recovered from biofilms formed on fragments of teat cup rubbers ranged from 4.64 to 6.87 log CFU/cm⁻², and from 3.61 to 5.70 log CFU/cm⁻² for the milk strains and the blood strains, respectively (Table V). Seven milk isolates developed more intense biofilms with a higher density of bacterial cell than the reference strain ATCC 19111 (5.80 log CFU/cm⁻²), whereas all

clinical strains were weaker biofilm formers than the reference strain (Fig. 4).

The biofilm formation intensity by *L. monocytogenes* isolates was confirmed under a confocal microscope (Fig. 5).

The correlation between antibiotic resistance, virulence genes, and biofilm formation ability. It was found that the strains with more virulence genes were resistant to a higher number of antibiotics, and their biofilms appeared to be more intense. There was a high positive correlation ($r=0.956$) and positive correlation ($r=0.896$) between the efficiency of biofilm formation, the number of virulence genes and the antibiotic resistance level of isolates derived from milk (Fig. 6) as well as clinical strains (Fig. 7), respectively. For the clinical isolates, only the correlation between the number of virulence genes and the efficiency of biofilm formation was not significant.

The spread of *L. monocytogenes* through contaminated teat rubbers. Transmission of *L. monocytogenes* on the skin of the udder and into the milk. Bacteria were transferred to skin pieces by the contact with the contaminated teat rubber (Table VI). The number of bacterial cells from the strains isolated from cow's milk that was detected in eight subsequent fragments of the udder skin in the number ranged from 1.08 log CFU/cm⁻² (M231) to 6.87 log CFU/cm⁻² (M372) (Table VI). Only the M63 strain was not isolated from the 8th fragment and its density was 1.59 log CFU/cm⁻² on the 7th fragment (Table VI). Clinical strains (B5 and B6) that developed strong biofilms were isolated

Table V
The intensity of biofilm formation by *L. monocytogenes* strains derived from cow's milk and the blood.

Strains origin	The strain identification number	Mean number of bacteria [log CFU/cm ²]	STD
Milk	372	6.87	± 6.12*
Milk	375	6.79	± 6.09
Milk	112	6.71	± 6.13
Milk	368	6.64	± 6.17
Milk	344	6.05	± 5.21
Milk	143	5.95	± 4.99
Milk	267	5.81	± 5.07
Milk	251	5.18	± 4.80
Milk	185	4.99	± 4.06
Milk	7m	4.94	± 4.00
Milk	199	4.94	± 3.76
Milk	317	4.89	± 3.78
Milk	4m	4.85	± 3.67
Milk	282	4.81	± 3.81
Milk	76	4.77	± 3.65
Milk	29	4.76	± 3.74
Milk	231	4.74	± 3.96
Milk	63	4.64	± 3.72
ATCC	19111	5.80	± 5.04
Blood	5	5.70	± 4.87
Blood	6	5.67	± 4.76
Blood	3	4.18	± 3.83
Blood	1	4.01	± 2.87
Blood	8	4.00	± 3.27
Blood	2	3.97	± 3.61
Blood	9	3.92	± 3.83
Blood	7	3.72	± 3.63
Blood	10	3.71	± 3.72
Blood	4	3.61	± 3.71

* - Standard deviation

from seven consecutive fragments of the skin and the number of bacterial cells was 1.26 log CFU/cm² for the strain B6 and 1.43 log CFU/cm² for the strain B5. The clinical isolates that developed weak biofilms were detected in four consecutive fragments and the number of bacterial cells was 1.20 log CFU/cm² for the strain B4, and 1.49 log CFU/cm² for the strain B10 (Table VI). Strain ATCC 19111 was transferred from the teat rubber to seven consecutive fragments of the skin and the number of cells on the last one was 1.53 log CFU/cm² (Table VI). It was also shown that the contamination level of the first skin fragment was higher for the strains that formed a weaker biofilm (Table VI).

The obtained results showed that even if *L. monocytogenes* was not detected on the fragment of the udder

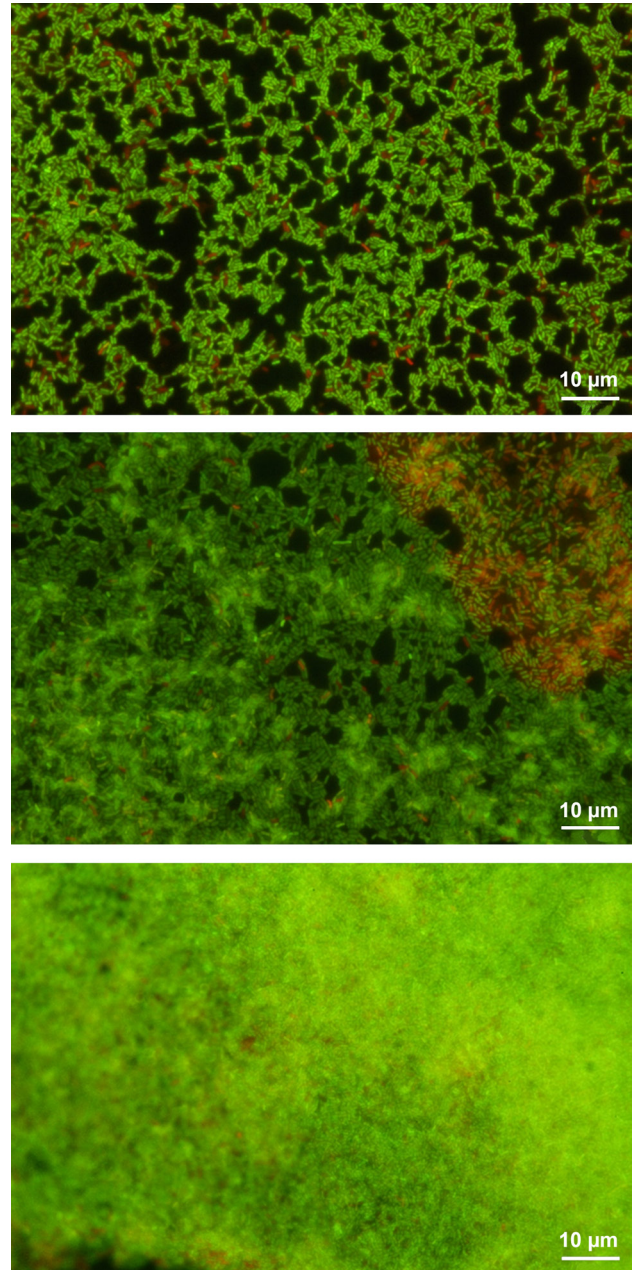


Fig. 5. The intensity of biofilm formation by the selected *L. monocytogenes* strains.

A - weak biofilm, B - moderate biofilm, C - strong biofilm, green - live cells, red - death cells.

skin (the number of bacteria was below the detection threshold), the bacteria were transferred from the teat rubber and their number increased to 1.38–2.92 log CFU/cm² along with incubation time (Table VI).

All *L. monocytogenes* strains that developed biofilms on teat rubbers also caused contamination of milk flowing through these rubbers. The number of bacteria recovered from milk ranged from 2.71 to 3.37 log CFU/ml for milk strains, and from 2.57 to 2.94 log CFU/ml for clinical strains (Table VII). The reference strain was grown in milk to the density of 2.97 log CFU/ml (Table VII).

Multiple correlation (x/y/z): 0.9563 (p=0.0001)
 Correlation (x/y): 0.9416 (p=0.0001)
 Correlation (x/z): 0.8912 (p=0.0001)
 Correlation (y/z): 0.9560 (p=0.0001)

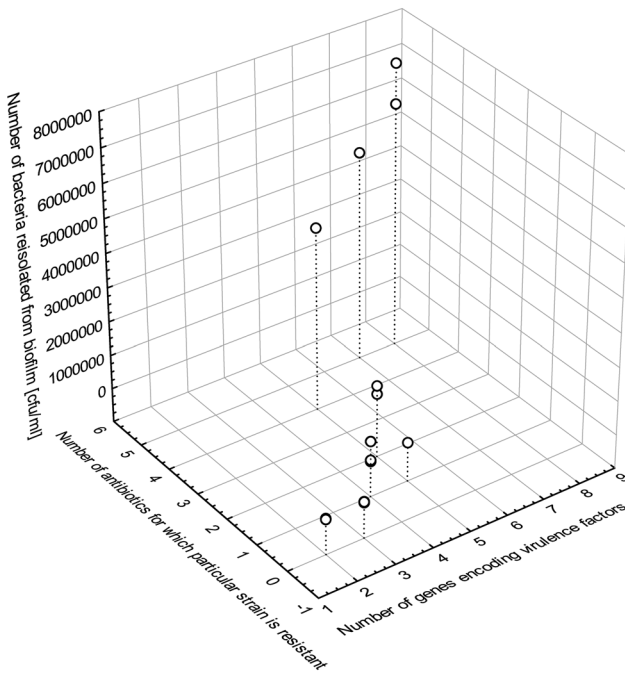


Fig. 6. Multiple correlations between the intensity of biofilm formation, the number of virulence genes, and the drug resistance of *L. monocytogenes* strains isolated from milk.

Multiple correlation (x/y/z): 0.8963 (p=0.0034)
 Correlation (x/y): 0.8154 (p=0.0040)
 Correlation (x/z): 0.4202 (p=0.2266)
 Correlation (y/z): 0.8010 (p=0.0054)

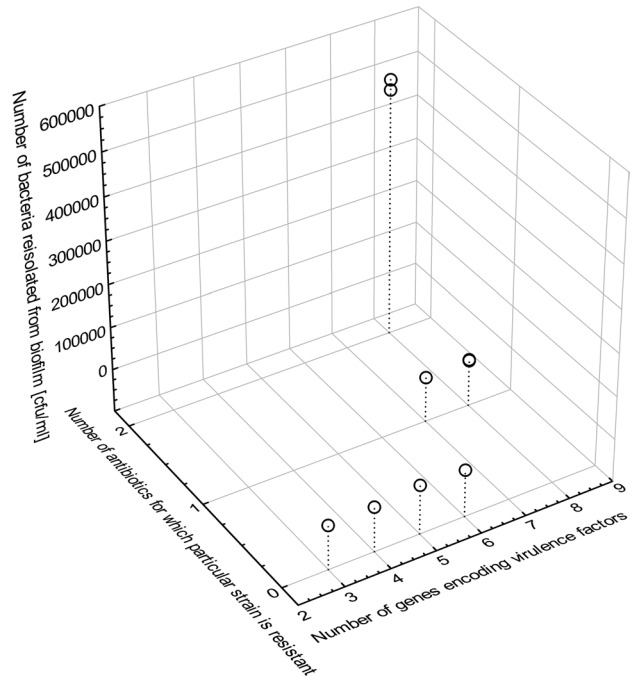


Fig. 7. Multiple correlations between the intensity of biofilm formation, the number of virulence genes, and the drug resistance of the clinical *L. monocytogenes* strains.

Table VI
 Transmission of *L. monocytogenes* from a biofilm on silicone teat cups to the cow's udder skin.

Strain	Number of re-isolated <i>L. monocytogenes</i> strains [log CFU/cm ²]									
	Silicone teat cup	Udder skin								Regrowth on first negative or last positive sample (12 h/25°C)
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8		
M372	6.87* ± 6.12**	4.33 ± 3.98	3.88 ± 3.77	3.71 ± 3.34	3.45 ± 2.88	2.92 ± 2.71	2.66 ± 2.35	2.09 ± 2.31	1.85 ± 1.84	2.92 ± 2.74
M375	6.79 ± 6.09	4.54 ± 4.37	3.99 ± 3.82	3.65 ± 3.63	3.10 ± 3.45	2.83 ± 2.69	2.51 ± 1.96	1.92 ± 1.82	1.61 ± 1.31	2.79 ± 2.62
M231	4.74 ± 3.96	3.87 ± 3.72	3.76 ± 3.79	3.47 ± 3.55	2.79 ± 2.71	2.52 ± 2.33	1.93 ± 1.20	1.67 ± 1.47	1.08 ± 1.54	1.99 ± 1.85
M63	4.64 ± 3.72	3.96 ± 3.82	3.80 ± 3.33	3.34 ± 3.62	2.70 ± 2.58	2.33 ± 2.32	1.86 ± 1.78	1.59 ± 1.48	n.d.	1.87 ± 1.51
ATCC 19111	5.80 ± 5.04	3.64 ± 3.67	3.02 ± 3.51	2.79 ± 2.39	2.62 ± 2.44	2.14 ± 2.48	1.79 ± 1.51	1.53 ± 1.63	n.d.	2.01 ± 2.31
B5	5.70 ± 4.87	3.56 ± 3.71	2.85 ± 2.70	2.63 ± 2.20	2.48 ± 2.23	1.96 ± 1.86	1.76 ± 1.61	1.43 ± 1.01	n.d.	1.81 ± 1.20
B6	5.67 ± 4.76	3.47 ± 3.17	2.91 ± 2.76	2.76 ± 1.99	2.39 ± 2.61	1.92 ± 1.65	1.64 ± 1.71	1.26 ± 1.33	n.d.	1.72 ± 1.80
B10	3.71 ± 3.72	2.85 ± 2.58	2.57 ± 2.20	1.88 ± 1.57	1.49 ± 1.04	n.d.***	n.d.	n.d.	n.d.	1.63 ± 1.41
B4	3.61 ± 3.71	2.92 ± 2.88	2.73 ± 2.53	1.84 ± 1.61	1.20 ± 1.35	n.d.	n.d.	n.d.	n.d.	1.38 ± 1.50

* - Mean, ** - Standard deviation, *** - Not detected

Table VII
Transmission of *L. monocytogenes* from a biofilm on silicone teat cups to milk.

Strain	Number of re-isolated <i>L. monocytogenes</i>	
	Silicone teat cup [log CFU/cm ²]	Milk [log CFU/ml]
M372	6.87* ± 6.12**	3.37 ± 3.03
M375	6.79 ± 6.09	3.29 ± 3.37
M231	4.74 ± 3.96	2.75 ± 2.62
M63	4.64 ± 3.72	2.71 ± 2.52
ATCC 19111	5.80 ± 5.04	2.97 ± 2.85
B5	5.70 ± 4.87	2.94 ± 2.74
B6	5.67 ± 4.76	2.84 ± 2.32
B10	3.71 ± 3.72	2.63 ± 2.51
B4	3.61 ± 3.71	2.57 ± 2.32

* – Mean, ** – Standard deviation

Transmission of L. monocytogenes in udder – teat rubber – udder model. It was shown that *L. monocytogenes* found on the udder skin may be transferred to

sterile teat rubber and then can cause the contamination of sterile udder skin (Table VIII). For all strains tested, the number of bacteria on the skin ranged from 5.13 to 5.91 log CFU/cm⁻², irrespective of their origin (milk vs. human) (Table VIII). Bacteria from the skin fragments were transferred to the teat rubber and the number of the bacteria reisolated ranged from 3.58 to 3.81 log CFU/cm⁻². In turn, the contact of the teat rubber with six consecutive sterile pieces of udder skin resulted in the transmission of *L. monocytogenes* to from four (for strains No M63, B4, and B10) to six (for strains M372, M231, ATCC 19111, and B6) skin fragments (Table VIII). The number of bacteria isolated from the last skin fragment examined was from 1.11 to 1.92 log CFU/cm⁻² (Table VIII). This may suggest the possibility of more than six consecutive udders contamination.

To confirm *L. monocytogenes* proliferation on skin fragments, the fragments were incubated 12 hours at 25°C. It appeared that bacteria were detected in both cases. Moreover, the increase in their numbers on the last tested fragments was observed (Table VIII).

Discussion

Since *L. monocytogenes* is widespread in the environment and survives in harsh environmental conditions, it may easily contaminate food (Walecka 2011).

Table VIII
Listeria spp. transmission system: udder skin – silicone teat cup – udder skin.

Strain	Number of re-isolated <i>L. monocytogenes</i> [log CFU/cm ²]								
	Silicone teat cup	Udder skin	Udder skin						Recovery of bacteria (12 h/25°C)
			Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	
M372	5.91* ± 5.81**	3.81 ± 3.61	3.77 ± 3.73	3, 0 ± 2.85	3.00 ± 2.85	2.81 ± 2.65	2.81 ± 2.65	1.92 ± 1.82	2.83 ± 2.53
M375	5.84 ± 5.63	3.68 ± 3.45	3.61 ± 3.83	2.68 ± 2.50	2.68 ± 2.50	1.87 ± 1.93	1.87 ± 1.93	n.d.***	1.66 ± 1.76
M231	5.57 ± 5.60	3.71 ± 3.23	3.66 ± 3.54	2.68 ± 2.72	2.68 ± 2.72	2.00 ± 1.90	2.00 ± 1.90	1.11 ± 1.16	2.37 ± 2.48
M63	5.52 ± 5.34	3.54 3.63	3.47 ± 3.55	1.47 ± 1.66	1.47 ± 1.66	1.00 ± 1.69	1.00 ± 1.69	n.d.	1.26 ± 1.03
ATCC 19111	5.66 ± 5.54	3.76 ± 3.57	3.72 ± 3.27	2.74 ± 1.99	2.74 ± 1.99	2.26 ± 1.98	2.26 ± 1.98	1.64 ± 1.47	2.57 ± 2.32
B5	5.71 ± 5.61	3.62 ± 3.20	3.56 ± 3.47	2.57 ± 2.42	2.57 ± 2.42	1.84 ± 1.03	1.84 ± 1.03	n.d.	1.46 ± 1.58
B6	5.69 ± 5.45	3.79 ± 3.86	3.73 ± 3.70	2.91 ± 2.78	2.91 ± 2.78	2.72 ± 2.64	2.72 ± 2.64	1.81 1.71	2.72 ± 2.63
B10	5.32 ± 5.68	3.59 ± 3.02	3.53 ± 3.37	1.62 ± 1.48	1.62 ± 1.48	1.11 ± 1.46	1.11 ± 1.46	n.d.	1.20 ± 1.68
B4	5.13 ± 5.40	3.58 ± 3.50	3.51 ± 3.58	1.52 ± 1.61	1.52 ± 1.61	0.90 ± 0.86	0.90 ± 0.86	n.d.	1.08 ± 1.71

* – Mean, ** – Standard deviation, *** – Not detected

L. monocytogenes is isolated from both unpasteurized and pasteurized cow's milk, dairy products such as soft cheeses, and dairy farms (Fleming et al. 1985; Van Kessel et al. 2004; CDC 2007; Fox et al. 2009; Lomanco et al. 2009; Balandyte et al. 2011; Van Kessel et al. 2011). In recent years, such products have been linked to several outbreaks of listeriosis (Rocha et al. 2013; CDC 2014; CDC 2016). Although milk products have repeatedly proved to be the source of this pathogen, the characteristics of *L. monocytogenes* strains isolated from unpasteurized milk and dairy farms remain unclear.

In the present study, *L. monocytogenes* was isolated from 21 (5.5%) out of 380 milk samples. Similar results were obtained in the Czech Republic (3.2%) (Gelbíčová and Karpíšková 2012a), Ethiopia (4.0%) (Garedew et al. 2015), England, Wales, India (5.1%) (Siegman-Igra et al. 2002; Kalorey et al. 2008), Iran (5.4%) (Jamali and Radmehr 2013), and Spain (6.5%) (Vilar et al. 2007). In contrast, *L. monocytogenes* strains were isolated only three times out of 294 milk samples in Sweden (Waak et al. 2002). In another study carried out in India, the occurrence of *L. monocytogenes* in fresh milk was considerably higher and accounted for 21.73% (Sharma et al. 2012).

Our studies showed the presence of the *hlyA* gene in all strains tested, regardless of their origin (milk vs. human). This is in accordance with the study of Indrawattana et al. (2011) and Aurora et al. (2008). In the present study, we found the *plcB* gene in 100% of the strains isolated from milk and in 80% of clinical isolates. This gene was detected in all strains of *L. monocytogenes* in the studies of Gelbíčová and Karpíšková (2012b), Mureddu et al. (2014), and Wieczorek and Osek (2017). Strains without the *plcB* gene divide but have reduced the ability to escape from phagosome (Bielecki 1994). We showed the presence of the *iap* gene in six (33.3%) strains cultured from milk and seven (70.0%) isolates from blood. In turn, Al-Nabulsi et al. (2015) confirmed the presence of the *iap* gene only among 16.6% of *L. monocytogenes* strains isolated from processed meat whereas Mureddu et al. (2014) demonstrated its presence in 97.1% strains of this species. Spontaneous mutants with reduced secretion of p60 protein do not lose their ability to grow intracellularly and the mutation is easily reversible. In contrast, insertional mutations in the region of the *iap* gene significantly reduce the hemolytic activity of *L. monocytogenes* (Rocourt et al. 2000).

To date, many studies evaluating the drug susceptibility of *L. monocytogenes* have been conducted (Morvan et al. 2010; Rahimi et al. 2010; Dalzini et al. 2016). Since the isolation of the first drug-resistant *L. monocytogenes* strains a systematic growth in resistance of these bacteria to antimicrobial agents has been observed (Poyart-Salmeron et al. 1990; Srinivasan et al. 2005; Morvan et al. 2010; Pesavento et al. 2010; Jamali

et al. 2013). Therefore, it is important to monitor drug resistance of this pathogen. In the present study, eight (44.4%) isolates from milk and four (40.0%) isolates from blood were susceptible to all the antibiotics tested. On the contrary, most (98.2%) of *Listeria* spp. strains isolated from cow's milk and products thereof showed resistance to at least one antibiotic in Iran (Rahimi et al. 2010). In our study, the greatest number of *L. monocytogenes* strains from milk was resistant to penicillin (44.4%), followed by erythromycin (33.3%), cotrimoxazole (27.7%), and ampicillin (22.2%). Similar results were obtained by Srinivasan et al. (2005) and Pesavento et al. (2010). A much higher percentage of the resistant strains was reported by Jamali et al. (2013), who isolated *L. monocytogenes* strains from the milk of cows without signs of mastitis and from milk of cows with the clinical form of mastitis. In our study, four (40.0%) strains isolated from humans were resistant only to erythromycin, whereas two (20.0%) strains were resistant to meropenem and erythromycin. These results are similar to those obtained in other countries (Marco et al. 2000; Vitas et al. 2007; Prieto et al. 2016). In this study, we also found that almost half of the strains examined (eight, 44.4%) isolated from milk are resistant to penicillin, while no penicillin-resistant *L. monocytogenes* isolated from blood samples was observed. It may be due to the recommended empiric use of penicillins in the treatment of contagious disease, including mastitis, in ruminants (EVIRA 2018).

We found also three (16.7%) strains isolated from milk resistant to all of the antibiotics tested. The strain is described as MDR if is resistant to at least three various groups of antibiotic. According to this definition, we found four (22.2%) MDR strains among strains isolated from milk, and none MDR strain was isolated from blood samples. Similar results were shown by Garedew et al. (2015) and Pesavento et al. (2010), who isolated four (16.7%) and 11 (27.5%) multidrug-resistant strains from food, respectively. In contrast, in the study by Jamali et al. (2013) as much as 71.4% of multidrug-resistant strains of *L. monocytogenes* from milk were detected. Charpentier and Courvalin (1999) have shown that the extensive use of antimicrobials in animal production, as well as clinical treatment of animals and humans, contributed to the continuous evolution of antimicrobial-resistant (AMR) bacteria with a diverse pool of genetically-transferred resistance determinants. Thereby, food of animal origin may be a source of AMR *L. monocytogenes* strains. In the food processing environment, *L. monocytogenes* may face many adverse conditions such as heat, high pressure, irradiation, acids, salts, and oxidants which induce the cross-protection against the same or other types of stresses (Wesche et al. 2009). It was shown, that the exposure of *L. monocytogenes* isolates derived from

food to variable pH, cold, heat and salt stress, disinfectants, and low water activity increased their resistance to various antibiotics (Beuls et al. 2012; Al-Nabulsi et al. 2015; Faezi-Ghasemi and Kazemi 2015). Clinical strains usually are not subjected to such stresses. This may explain the difference in AMR between clinical and food isolates.

In Poland, among 471 *L. monocytogenes* strains isolated from different foods and food-related sources from 2004 to 2010, no resistance to ampicillin, amoxicillin, chloramphenicol, erythromycin, gentamicin, rifampicin, sulfamethoxazole, trimethoprim, and vancomycin was reported (Korsak et al. 2012). On the contrary, a Lebanese study assessing AMR in *L. monocytogenes* recovered from traditional dairy products showed that all isolates (n=30) were resistant to at least one antimicrobial, including the resistance to ampicillin (60%), penicillin (90%), erythromycin (27%), gentamicin (7%), and SXT (17%) (Harakeh et al. 2009).

Monitoring of the occurrence of *L. monocytogenes* in food is a great challenge for food processing plants. The ability of these microorganisms to survive in a moist, cool environment and to form biofilm makes them difficult to eradicate. *L. monocytogenes* presence in milk and milk products may be caused by improper sanitization of surroundings and equipment used during milking, as well as ineffective disinfection in dairies (Tompkin et al. 2002). The present study evaluated the ability of the strains tested to form biofilm on silicone teat cups rubbers. Among *L. monocytogenes* strains derived from milk, seven isolates developed more intense biofilms than the reference ATCC® 19111™ strain. All clinical strains formed biofilm less intense than milk isolates ($p=0.005$). Latorre et al. (2010) isolated a considerable number of bacteria *L. monocytogenes* from milking machines. Using SEM micrography, they found that the bacteria were particularly visible in scratches on the inside plastic surface of the teat cups. In the study by Doijad et al. (2015), only nine out of 98 strains isolated from different environments formed robust biofilms. It is worthy to note that all strains that developed intense biofilms were isolated from milk and dairy products. In previous studies, it has been proved that *L. monocytogenes* can form biofilm on many surfaces such as polystyrene, polypropylene, glass, stainless steel, quartz, marble, and granite (Silva et al. 2008). Though, studies by Djordjevic et al. (2002) and Harvey et al. (2007) indicated that *L. monocytogenes* formed only weak or moderate biofilm on various surfaces. In turn, the study by Sinde et al. (2000) showed better adherence of *L. monocytogenes* to rubber when compared to stainless steel.

Bacteria within biofilms are much more resistant to antimicrobial agents than floating counterparts (Gong et al. 2013). To understand the relationship between biofilms and antimicrobial resistance in *L. monocyto-*

genes, we assessed biofilm formation ability of *L. monocytogenes* isolates together with their antimicrobial resistance. Our results showed very high positive correlation ($r=0.96$) for isolates from milk and high positive correlation ($r=0.80$) for clinical isolates. Such a relationship between biofilm production and antibiotic resistance was demonstrated for *Salmonella Pullorum* and uropathogenic *E. coli* (Adetunji et al. 2008; Gong et al. 2013). Adetunji et al. (2008) showed that strains of *L. monocytogenes* producing intense biofilms were more virulent and drug-resistant. Intrinsic resistance of biofilm bacteria is related to the presence of persister cells or efflux pumps that remove antibiotics from the biofilm environment (Korsak et al. 2005; Sauvage et al. 2008). Bacterial cells from deeper layers of biofilm are protected against antimicrobials by the upper layers. The penicillin resistance among the strong-biofilm forming *L. monocytogenes* strains may be correlated with the presence of PBP5 that binds penicillin G. This protein is a DD-carboxypeptidase related with the membrane fraction and lateral wall growth of *L. monocytogenes* cell (Korsak et al. 2005; Sauvage et al. 2008).

Our study also revealed a positive correlation between the number of virulence genes, drug resistance, and biofilm formation ability. This is in agreement with the study by Soni et al. (2013), which indicated that multi-drug resistant *L. monocytogenes* strains derived from clinical specimens, water, and milk possess a large number of virulence genes (*inlA*, *inlC*, *plcA*, *prfA*, *actA*, *hlyA*, and *iap*).

Our study showed that teat cups, contaminated during milking might contribute to the transmission of *L. monocytogenes* to cow's udders and finally to the milk. It was found that this phenomenon might affect at least several successively milked animals. This supports the study of Benić et al. (2012), which demonstrated that bacteria might be transmitted by utensils used during milking, mainly by the teat cups. It was also confirmed by Azevedo et al. (2016) studies, which found that improper hygiene of teat cups could cause transmission of *Staphylococcus* spp. both between the animals and to the milk tank.

A better understanding of the epidemiology of *L. monocytogenes* infections and the factors affecting their survival, spread and resistance is necessary to prevent contamination in the food industry and transmission of the pathogen. This may help in limiting listeriosis incidence in humans.

Conclusions

In the available literature, there are few data on the bacterial transmission *via* teat cups and the risks posed by this equipment. This study showed that

L. monocytogenes isolates originating from cow's milk are more resistant to antibiotics than the clinical strains. The most frequent virulence genes detected were *hlyA* and *plcB* among milk strains and *hlyA* and *prfA* in strains derived from the blood. The intensity of the biofilm formation was strain-dependent and was significantly higher in the milk strains. The association between biofilm formation the number of virulence genes, and antimicrobial resistance of *L. monocytogenes* strains was high positive for the isolates from milk ($r=0.96$) as well as for the clinical isolates ($r=0.90$). Our study showed also that teat cups, contaminated during the milking process, might play an important role in the transmission of *L. monocytogenes* to the milk, posing risk to the consumer health. For these reasons, it is reasonable to monitor incidence, susceptibility and biofilm formation by *L. monocytogenes* during milking and milk processing (especially unpasteurized milk).

Author's contributions

Krzysztof Skowron – development of the concept of experiments and methodology of research, collecting the materials for research, participation in the performance of the laboratory part of experiments, collection, and analysis of results, participation in the writing of the manuscript, management of the research team and preparing the publication

Ewa Wałęcka-Zacharska – development of the concept of experiments and methodology of research, translation of the manuscript text

Katarzyna Grudlewska – participation in the performance of the laboratory part of experiments and writing of the manuscript

Natalia Wiktorczyk – participation in the performance of the laboratory part of experiments and the collection of materials for research and references

Agnieszka Kaczmarek – participation in the performance of the laboratory part of experiments and the collection of references

Grzegorz Gryń – the collection and analysis of results

Joanna Kwiecińska-Piróg – participation in the performance of the laboratory part of experiments, collection of materials for research, adaptation of the manuscript to the editorial requirements

Klaudia Juszczyk – participation in the performance of the laboratory part of experiments

Zbigniew Paluszak – obtaining funds for conducting research

Katarzyna Kosek-Paszowska – an adaptation of the manuscript to the editorial requirements

Eugenia Gospodarek-Komkowska – verification of the manuscript and final acceptance, obtaining funds for conducting research

All authors contributed to the draft of the manuscript and discussed results. All authors gave final approval for publication.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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