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Drug-susceptibility, biofilm-forming ability and biofilm survival on stainless steel of *Listeria* spp. strains isolated from cheese

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ABSTRACT

The aim of the study was to analyze the contamination of mold cheese (Brie, Camembert, Gorgonzola, Munster and Roquefort) with *Listeria* spp. and assessment of culturable cells number recovered from the biofilm formed on the surface of stainless steel by obtained strains. Identified isolates (MALDI TOF MS technique) were subjected to susceptibility testing (disk-diffusion method) and their genetic similarity (PFGE method), ability to form biofilm (quantitative method), biofilm dry weight, and biofilm survival on stainless steel were evaluated. Out of 250 samples of cheese 26 (10.4%) were *Listeria* spp. positive, including 15 isolates (6.0% of samples) of *L. monocytogenes*, 7 isolates of *L. innocua* (2.8% of samples) and 4 isolates of *L. welshimeri* species (1.6% of samples). Of the 26 isolates tested, 22 strains were genetically different. It was shown that *L. innocua* and *L. welshimeri* strains were sensitive to all antibiotics tested, while two (16.7%) *L. monocytogenes* strains were resistant to penicillin and one (8.3%) to erythromycin. *L. monocytogenes* formed biofilm most intensively on stainless steel, while *L. welshimeri* the least effectively. The median of bacteria number recovered from the biofilm for *L. monocytogenes* was 6.81 log CFU × cm^{-2}, for *L. innocua* – 5.63 log CFU × cm^{-2}, and for *L. welshimeri* – 4.93 log CFU × cm^{-2}. The survival in the biofilm of *Listeria* spp. strains decreased along with the increase in a storage temperature of steel coupons. The longest survival time was reported at 4 °C, i.e. 47.58–124.41 days, with an elimination rate of 0.06–0.13 log CFU × day^{-1}. Collectively, *L. monocytogenes* is the most prevalent species of *Listeria* genus in the mold cheese. The ability of *L. monocytogenes* strains to form biofilm on stainless steel and survive in the food processing environment increases chance of the secondary contamination of food posing risk to the consumer health.

1. Introduction

An increase in the frequency of food-borne diseases has been reported in the recent years. *Listeria* spp. is Gram-positive bacteria, able to grow at wide temperature and pH range as well as high NaCl concentration (Melo et al., 2015). The bacteria are widespread in the environment, which fosters the contamination of various food products (Korsak and Szuplewska, 2016; Şanlıbaba et al., 2018). The genus *Listeria* currently includes 17 species (Korsak and Szuplewska, 2016) of which *L. monocytogenes* is considered to be the main etiological factor of listeriosis (pathogenic to humans and animals) and *L. ivanovii* and *L. seeligeri* are sporadically isolated from humans (Sarfraz et al., 2017). Infections might be manifested by inflammation of the stomach and intestines (non-invasive form), meningitis, miscarriage and perinatal infections (invasive form) There is a systematic increase in the number of patients with confirmed listeriosis (EFSA, 2018; ECDC, 2018; Şanlıbaba et al., 2018). In 2017 2480 patients were diagnosed with listeriosis and 227 confirmed deaths were reported in the European Union (EFSA, 2018; ECDC, 2018). Moreover between 2017 and 2018 two outbreaks of listeriosis in Australia and Republic of South Africa, including 20 cases (8 deaths) and 1024 cases (200 deaths), respectively were reported (The Health Department of Republic of South Africa, 2018; WHO, 2018). An additional, very dangerous phenomenon, observed in recent years, is the increasing antibiotic resistance of *Listeria* spp. (Karadal and Yıldırım, 2014). According to Lungu et al. (2011), *L. monocytogenes* acquire antibiotic resistance genes due to horizontal

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gene transfer, of which some may be derived from commensal microorganisms found in food (Lungu et al., 2011).

Epidemics and single cases of listeriosis were reported after consumption of contaminated milk, soft cheese, undercooked meat, fish and unwashed raw vegetables (Meshref et al., 2015). Dairy products were related to both invasive and non-invasive listeriosis forms. In 2013 (6 cases, 1 death) and 2017 (8 cases, 2 deaths) two listeriosis outbreaks associated with cheeses were reported in USA (CDC, 2013; CDC, 2017). The Federal Agency for the Safety of the Food Chain (FASFC) (2011) reported that *L. monocytogenes* was present in 2.2–10.2% of raw milk samples in Europe. According to the European Food Safety Authority (EFSA) the occurrence of *L. monocytogenes* in goat, sheep and cow soft and semi-soft cheeses made from raw or low-heat-treated milk was significantly higher (2.4%) compared with cheeses made from pasteurized milk (0.5%) (EFSA, 2018; ECDC, 2018). Although, nowadays, most of cheeses worldwide are prepared from pasteurized milk, some researchers indicate that they can be equally dangerous in aspect of microbial contamination. Based on seven EFSA reports, covering the period 2005–2015, Martinez-Rios and Dalgaard (2018) found that there is no significant difference in the incidence of *L. monocytogenes* between raw milk and soft/semi-soft cheeses made from pasteurized milk. Moreover, soft cheeses produced from pasteurized milk tend to be more susceptible to microbial contamination. This may be due to the fact that endogenous microbiota of raw milk, including *Lactobacillus* spp. can play an inhibitory role in the development of *L. monocytogenes* (Schvartzman et al., 2011; Tiwari et al., 2014). Listeriosis incidents may be related to the consumption of both cheese prepared from pasteurized and non-pasteurized milk (Gould et al., 2014).The soft cheese is a product with a pH of 4.5–6.5 and NaCl content of 2.3–3.5%. These conditions do not inhibit the growth of *L. monocytogenes* posing a serious risk for the consumer health (Melo et al., 2015). The soft cheeses (mainly moldy and long-ripening ones, such as Brie, Camembert, Gorgonzola, Munster and Roquefort) are most frequently contaminated with *L. monocytogenes* (Choi et al., 2016; EFSA, 2018; ECDC, 2018; Lahou and Uyttendaele, 2017). The presence of other *Listeria* species in these cheeses, including *L. innocua* was reported (Angelidis et al., 2012). A significant proportion of microbial cheese contamination is associated with a lack of hygiene during cooling, storage and cutting of cheese. At this stage, the cleanliness of objects and surfaces (e.g. stainless steel) that have a contact with cheese plays an important role (Bernini et al., 2016). The flagship example of the important role of the production environment contamination in the cross-contamination of cheese is the Gorgonzola cheese case. Although pasteurization of milk inactivates the pathogens, the final product might be recontaminated e.g. during maturation (Cocolin et al., 2009). The contamination of the outer parts of the cheese is the most probable and in order to avoid the risk of *L. monocytogenes* the Consortium for the Protection of Gorgonzola Cheese found the cheese skin to be inedible (UE Notice 2008/C 111/17). However, it should be noticed, that *L. monocytogenes* may still be transferred into the interior of the cheese during portioning, slicing and packaging, being a threat to the consumer (Bernini et al., 2015). Cross-contamination is an important aspect related to food contamination with *Listeria* spp. (Melo et al., 2015). The adhesion of *Listeria* spp. strains to biotic surfaces and, consequently, the emergence of biofilm is widely documented, e.g. in meat and dairy processing (Unnerstad et al., 1996; Simões et al., 2010). Bacteria in the biofilm structure are more resistant to disinfectants and UV radiation as compared to planktonic cells, which makes it difficult to eliminate them from food plants (Davidson and Harrison, 2002). One of the materials, most commonly used in the food industry, is stainless steel (Simões et al., 2010; Bernini et al., 2016). It is estimated that cross-contamination related to surfaces concerns 39% of outbreaks of food-borne diseases (Evans et al., 1998). In cheese production plants, the presence of *L. monocytogenes* has been demonstrated both on equipment used in the processing stage, in direct contact with the product (storage tanks, conveyor systems/chains, table tops, milk filler, brine pre-filtering device, shelves used to store cheese during maturation, washing equipment and washing solution used in soft cheeses), as well as in the post-processing environment (ripening, refrigerating/freezing) (Pritchard et al., 1995). Biofilms can be formed on all these surfaces, especially when they are not properly washed and there are residues of organic matter on them (Kousta et al., 2010). For this reason, it is important to assess the biofilm formation ability on the steel surface and bacterial survival in biofilms at temperatures corresponding to the conditions during the cheese processing.

The aim of this study was to identify and evaluate the occurrence frequency, drug susceptibility, dry weight of biofilm, number of culturable cells recovered from the biofilm and survival on stainless steel fragments of *Listeria* spp. strains isolated from five types of blue cheese.

2. Material and methods

2.1. Material

The analyzed material consisted of 250 samples of blue cheese. Fifty samples from five most popular types of cheese; i.e. Brie, Camembert, Gorgonzola, Munster and Roquefort; were collected. The cheeses were bought in randomly selected hypermarkets in Poland in two rounds with a two-month interval. Brie and Camembert cheeses were produced by one manufacturer and Gorgonzola, Munster and Roquefort by another. For the research cheeses with a minimum 2/3 of the shelf-life period were used.

2.2. Isolation of Listeria spp. from cheese

To isolate *Listeria* spp. from cheese, 10 g of sample was transferred to 90 cm^3 half-Fraser broth (Merck), homogenized in a laboratory paddle blender BagMixer 400 CC (Bionovo) and incubated for 24 h at 30 °C. Then, 0.1 cm^3 of the culture was transferred into 10 cm^3 Fraser broth (Merck) and the secondary selective enrichment was performed. After 48 h at 37 °C culture was plated onto selective ALOA agar (Merck) and incubated for 24 h at 37 °C.

2.3. Identification of strains isolated from cheese

Colonies suspected of belonging to the *Listeria* genus (according the morphological traits on ALOA) were transferred onto Columbia Agar with 5% Sheep Blood (Becton Dickinson), incubated at 37 °C for 24 h and used for final identification. The final identification was based on MALDI-TOF Mass Spectrometry technique. The ethanol-formic acid extraction procedure was applied for samples preparation as described Freiwald and Sauer (2009). The acquisition and analysis of mass spectra were performed by a Microflex LT mass spectrometer (Bruker) using the MALDI Biotyper software package (version 4.1) with the reference Bruker Taxonomy database (Bruker) and default parameter settings as published previously by Schulthess et al. (2013). The Bruker bacterial test standard (Bruker) was used for calibration according to the instructions of the manufacturer. The standard Bruker criteria for obtained identification score values interpretation are as follow: range 2.300…3.000 – highly probable species identification; range 2.000…2.299 – secure genus identification, probable species identification; range 1.700…1.999 – probable genus identification; range 0.000…1.699 – not reliable identification (standard Bruker cutoff score values proposed by system). All tested isolates were identified with score values > 2000, so it means that it was highly probable species identification or secure genus identification and probable species identification.

2.4. Genetic relation determination of Listeria spp. strains isolated from cheese

All isolates of the *Listeria* genus were subjected to genotyping using

Pulsed-Field Gel Electrophoresis (PFGE). The procedure was performed in accordance with the Standard Operating Procedure for PulseNet PFGE of *Listeria monocytogenes* (PNL04, April 2013) with modification. For DNA enzymatic digestion the FastDigest *ApaI* enzyme (Thermo Scientific) (37 °C/15 min) with FastDigest Green Buffer (Thermo Scientific) were used.

To assess genetic relation between the isolates phylogenetic dendrograms were drawn in the CLIQS 1D Pro (TotalLab) software. Data clustering was performed using the UPGMA hierarchic grouping technique with the Dice coefficient.

2.5. Drug susceptibility evaluation of strains tested

Evaluation of drug susceptibility was performed for genetically different isolates derived from cheese by using the disk diffusion method on the Mueller-Hinton agar with 5% defibrinated Horse Blood and 20 mg/L β-NAD (MH-F, bioMérieux). The disks with penicillin (1 IU), ampicillin (2 μg), meropenem (10 μg), erythromycin (15 μg) and cotrimoxazole (1.25–23.75 μg) were used. Antibiograms were incubated in the atmosphere enriched in 5% CO_2 at 35 °C for 18 h. The results were interpreted, according to the recommendations of EUCAST v. 8.0.

2.6. Evaluation of number of culturable cells recovered from the biofilm formed by Listeria spp. strains

Quantitative evaluation of number of culturable cells recovered from the biofilm formed by genetically unrelated *Listeria* spp. strains from cheese was performed on sterile stainless steel coupons (1 cm × 1 cm × 1 mm; AISI 304 type) as previously described (dos Reis-Teixeiraa et al., 2017).

Sterile steel coupons, three replications for each strain, were placed in tubes containing 3 cm^3 suspension of bacterial suspension in BHI (Merck) of a density of 0.5 (MacFarland scale) and incubated in the aerobic atmosphere at 37 °C for 72 h. The medium was replaced with a sterile one every 24 h. At each medium change, the coupons were rinsed with PBS (Phosphate Buffered Saline, BTL). Stainless steel fragments incubated in the sterile BHI medium were used as the negative control. After incubation, the samples were rinsed with PBS solution and placed in a tube containing 3 cm^3 of this solution. Next, sonication was performed using the Ultrasonic DU-4 (Nickel-Electro Ltd.) sonicator.

After sonication, serial 10-fold dilutions of the obtained suspension were prepared, plated on the Columbia Agar medium with 5% Sheep Blood (Becton Dickinson) and incubated for 24 h at 37 °C. The results were presented as the log CFU × cm^{-2}.

For the positive control a strong biofilm-forming *Staphylococcus aureus* ATCC 35556 was used and the negative control was sterile steel coupon placed in sterile BHI.

The results were averaged for each strain and compared with each other by using the analysis of variance (ANOVA) and the post-hoc Bonferroni test, at the significance level $\alpha = 0.05$. The above analyses were performed using the Statistica 12 PL (StatSoft) software.

2.7. Determination of biofilm dry weight

Determination of the dry weight of biofilm was carried out by a drying-weight method, as previously described by Gomes et al. (2011) and Meissner et al. (2013), whereby the biofilm was dried and weighed directly on the steel coupon on which it was formed. In the first stage, sterile stainless steel coupons were weighted and described with the appropriate numbers. For each strain tested 10 coupons were prepared. Then a biofilm was formed on these coupons as described in Section 2.6. After 72 h of biofilm formation, the coupons were washed with sterile PBS and dried at 56 °C for 2 h. After this time, the coupons were weighted again. Then, for each strain of *Listeria* spp., the average weight of sterile coupons and coupons with the formed and dried biofilm was calculated. Biofilm dry weights were calculated as the differences between these two measurements. As a negative control 10 sterile steel coupons were subjected to the procedure of biofilm formation but without addition of bacteria to sterile BHI at the first stage. Next, the coupons were washed with PBS, dried and weighted. The result was presented as a biomass of the biofilm per 1 cm^2 of steel coupon surface.

The results were averaged for each strain and compared with each other by using the analysis of variance (ANOVA) and the post-hoc Tukey test, at the significance level $\alpha = 0.05$. The above analyses were performed using the Statistica 12 PL (StatSoft) software. Moreover, the Pearson's correlation coefficient between the number of live bacteria isolated from the biofilm and the dry mass of the biofilm produced by a given strain was calculated and evaluated according to Guilford's scale.

2.8. Evaluation of Listeria spp. survival in biofilm on stainless steel

To evaluate the survival of *Listeria* spp. in the biofilm on stainless steel the strongest biofilm-forming strain of each species, isolated from each type of cheese was selected.

The biofilm of *Listeria* spp. on stainless steel coupons was produced in accordance with the methodology described in Section 2.6. Steel coupons with a 72-hour biofilm were individually placed in separate, sterile, tightly closed, plastic containers and stored at 4, 20 and 37 °C for 40 days. Every day, the sterile gauze soaked with 2 ml of sterile water was exchanged in each container, to ensure humidity. This procedure was performed in a sterile laminar chamber. To evaluate cell survival in the biofilm, one steel coupon was taken directly after finish the procedure of biofilm formation and after 1, 2, 3 and every 5 days for 40 days or till the number of bacteria decreased under detection limit. Each time the strains of *Listeria* spp. were re-isolated and counted, according to the procedure described in Section 2.6. The experiment was performed in triplicate for each strain tested.

Based on the obtained results, a simple linear regression was drawn and the theoretical survival time and elimination rate were calculated.

The results were averaged for each *Listeria* species and for each strain, and compared with each other using the analysis of variance (ANOVA) and the post-hoc Tukey test, at the significance level $\alpha = 0.05$. The analyses were performed using the Statistica 10 PL (StatSoft) software.

3. Results

The performed research showed that of the 250 cheese samples analyzed *Listeria* spp. was detected in 26 (10.4%) of them. Identification based on the MALDI-TOF MS technique allowed distinguishing three *Listeria* species. The *L. monocytogenes* (LMO) species included 15 (6.0% of samples) isolates, *L. innocua* (LIN) - 7 (2.8% of samples), and *L. welshimeri* (LWE) - 4 isolates (1.6% of samples). The most frequently *Listeria* spp. were isolated from Roquefort cheese (12, 46.2% - 7 LMO, 3 LIN and 2 LWE) subsequently from Camembert (6, 23.1% - 3 LMO, 1 LIN and 2 LWE), Munster (4, 15.4% - 3 LMO and 1 LIN), Brie (2, 7.7% - 2 LMO) and Gorgonzola cheeses (2, 7.7% - 2 LIN).

3.1. Genetic relation determination of Listeria spp. strains isolated from cheese

For *L. monocytogenes*, *L. innocua* and *L. welshimeri*; three, two and two major monophyletic branches were isolated, respectively (Fig. 1). The analysis of genetic similarity revealed 22 different genetic profiles among 26 *Listeria* isolates. As for *L. monocytogenes*, three isolates from Roquefort had the same genetic profile and two isolates from Munster were genetically identical. The LMO-R1 strain from Roquefort and LMO-C3 strain from Camembert cheese were also similar. On the other hand, *L. monocytogenes* strains isolated from Brie and Munster cheese, formed one monophyletic subgroup (Fig. 1). Two *L. innocua* isolates,

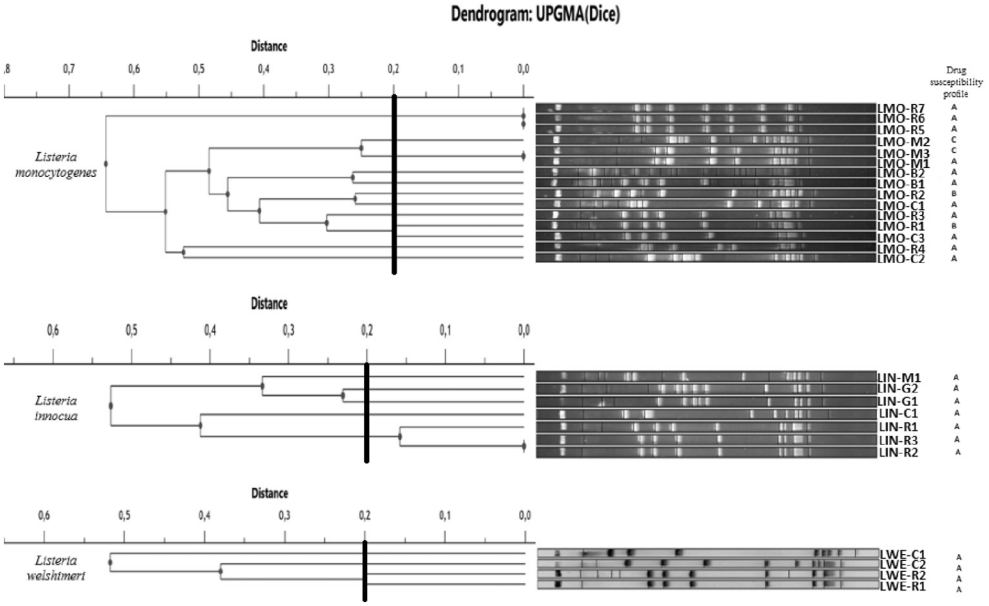

Fig. 1. Genetic similarity dendrogram of the collected *Listeria* spp. isolates (vertical black line separates strains considered as genetically similar).

derived from Roquefort cheese, represented the same genotype and together with the LIN-R1 strain created a single monophyletic subgroup. Moreover, *L. innocua* strains obtained from Gorgonzola also belonged to one subgroup (Fig. 1). None of the *L. welshimeri* isolates was genetically identical though strains derived from Roquefort cheese were closely related (Fig. 1).

3.2. Drug susceptibility evaluation of strains tested

All *L. innocua* and *L. welshimeri* strains were sensitive to all antibiotics tested. On the other hand, among the *L. monocytogenes* strains, two (16.7%) were resistant to penicillin and one (8.3%) to erythromycin (Table 1). Both penicillin-resistant strains were isolated from Roquefort cheese and the erythromycin-resistant one from Munster cheese.

3.3. Evaluation of culturable cells number recovered from the biofilm formed by Listeria spp. strains

The number of culturable cells recovered from the biofilm was species-, origin- and strain-dependent (Table 2). The greatest number of culturable cells recovered from the biofilm on stainless steel was reported for *L. monocytogenes* (Median = 6.81 log CFU × cm^{-2}), then for *L. innocua* (Median = 5.63 log CFU × cm^{-2}) and the smallest number for *L. welshimeri* (Median = 4.93 log CFU × cm^{-2}) (Fig. 2). Differences between all calculated medians were statistically significant ($p \leq 0.05$) (Fig. 2).

Among all *Listeria* species tested, the greatest number of culturable cells recovered from the biofilm was found in strains isolated from Roquefort cheese and was statistically different from the numbers for the other cheese types (Table 2).

3.4. Determination of biofilm dry weight

The dry weight of the biofilm varied depending on the *Listeria* spp. species and the strain origin. In the case of *L. monocytogenes*, it ranged from 2.99 to 5.01 mg × cm^{-2}, for *L. innocua* from 1.94 to 2.56 mg × cm^{-2}, and for *L. welshimeri* from 1.49 to 3.08 mg × cm^{-2} (Table 2). The dry weight of the biofilm was associated with the number of *Listeria* spp. culturable cells isolated from the biofilm. In the case of *L. monocytogenes* and *L. innocua* a strong positive correlation was found, and for *L. welshimeri* a very strong positive correlation was noted (Table 2).

Table 1
Results of drug susceptibility evaluation of the *Listeria* spp. strains tested.

Profile name	Drug susceptibility profile	Number of strains	Strain identification number
L. monocytogenes			
A	R: – S: P, AM, MEM, E, SXT	9	LMO-B1, LMO-B2, LMO-C1, LMO-C2, LMO-C3, LMO-M1, LMO-R2, LMO-R4, LMO-R5
B	R: P S: AM, MEM, E, SXT	2	LMO-R1, LMO-R3
C	R: E S: P, AM, MEM, SXT	1	LMO-M2
L. innocua			
A	R: – S: P, AM, MEM, E, SXT	6	LIN-C1, LIN-G1, LIN-G2, LIN-M1, LIN-R1, LIN-R2
L. welshimeri			
A	R: – S: P, AM, MEM, E, SXT	4	LWE-C1, LWE-C2, LWE-R1, LWE-R2

S - sensitive, R - resistant, P - penicillin, AM - ampicillin, MEM - meropenem, E - erythromycin, SXT-cotrimoxazole, LMO – *L. monocytogenes*, LIN – *L. innocua*, LWE – *L. welshimeri*, B - Brie, C - Camembert, G - Gorgonzola, M - Munster, R – Roquefort.

Table 2
Number of *Listeria* spp. bacilli recovered from a biofilm on stainless steel and biofilm dry weight.

Strain	Number of bacilli re-isolated from a biofilm [log CFU × cm^{-2}]	Biofilm dry weight [mg × cm^{-2}]	Pearson's correlation coefficient
L. monocytogenes			
LMO-B1	6.60^{a}	3.10^{c}	0.898
	*4.87**	*0.68*	($p \leq 0.01$)
LMO-B2	6.68^{a}	3.34^{c}	
	5.28	*0.77*	
LMO-C1	6.84^{a,b}	4.27^{a,b}	
	4.96	*0.91*	
LMO-C2	6.73^{a}	3.50^{b,c}	
	5.18	*0.67*	
LMO-C3	6.79^{a,b}	3.63^{b}	
	4.84	*1.03*	
LMO-M1	6.56^{a}	3.30^{c}	
	5.03	*1.11*	
LMO-M2	6.45^{a,d}	2.99^{c}	
	4.86	*0.92*	
LMO-R1	6.94^{b}	4.88^{a}	
	4.84	*1.13*	
LMO-R2	6.89^{b}	3.85^{b}	
	5.28	*0.80*	
LMO-R3	6.97^{b}	5.01^{a}	
	4.82	*0.84*	
LMO-R4	6.88^{b}	4.03^{a,b}	
	4.92	*1.07*	
LMO-R5	6.91^{b}	4.67^{a}	
	4.92	0.72	
L. innocua			
LIN-C1	5.72^{c,e}	2.29^{d}	0.817
	5.91	*0.49*	($p \leq 0.05$)
LIN-G1	5.27^{c,f}	2.00^{d}	
	3.93	*1.01*	
LIN-G2	5.39^{c,f}	2.17^{d}	
	4.20	*0.65*	
LIN-M1	5.55^{c}	1.94^{d}	
	4.20	*0.71*	
LIN-R1	5.85^{d,e}	2.56^{c,d}	
	4.53	*0.74*	
LIN-R2	5.77^{c,e}	2.40^{d}	
	4.13	*0.81*	
L. welshimeri			
LWE-C1	4.85^{f}	1.49^{e}	0.991
	3.18	*0.43*	($p \leq 0.01$)
LWE-C2	4.90^{f}	1.63^{d,e}	
	3.07	*0.62*	
LWE-R1	6.29^{a,d}	3.08^{c}	
	5.32	*0.50*	
LWE-R2	4.96^{f}	1.52^{e}	
	3.56	*0.70*	

a, b, c, … - values marked with different letters differ significantly; * - standard deviation; LMO – *L. monocytogenes*, LIN – *L. innocua*, LWE – *L. welshimeri*, B - Brie, C - Camembert, G - Gorgonzola, M - Munster, R - Roquefort.

3.5. Evaluation of *Listeria* spp. survival in biofilm on stainless steel

Based on the obtained results, it was found that all tested strains forming a biofilm were gradually eliminated from the surface of stainless steel. The elimination rate varied, depending on the storage temperature of the steel coupons and the *Listeria* spp. strains. Based on the simple regression equations, the theoretical survival time of *Listeria* spp. in the biofilm on steel and the bacteria daily elimination rate were calculated (Table 3). The theoretical survival time of *Listeria* spp., depending on the species and strain tested, ranged from 47.58 to 124.41 days at 4 °C, 29.54–64.74 days at 20 °C and 13.85–32.50 days at 37 °C, and elimination rate was of 0.06–0.13, 0.11–0.17 and 0.19–0.36 log CFU × day^{-1}, respectively (Table 3). Differences in theoretical survival resulting from temperature were statistically significant for *L. monocytogenes* and *L. innocua* strains, whereas for *L. welshimeri* strains

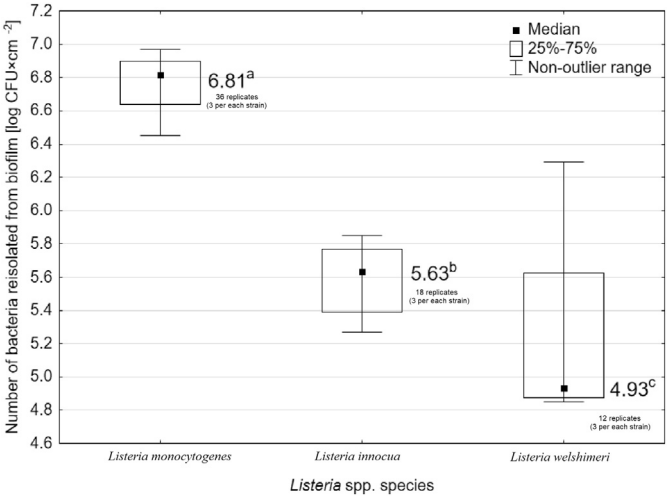

Fig. 2. Number of culturable *Listeria* spp. cells recovered from the biofilm, depending on the strain species (a, b, c - values marked with different letters differ statistically significantly ($p \leq 0.05$)).

these differences were significant at 4 °C and 37 °C (Table 3).

The longest theoretical survival time and the lowest elimination rate were found for *L. monocytogenes*, regardless of temperature (Table 3). In contrast, *L. welshimeri* strains survived the shortest and were the fastest eliminated. Differences in the survival between *Listeria* spp. strains were significant at 4 °C and 20 °C (Table 3).

Differences in the survival between individual strains within the tested species were statistically insignificant, regardless of the coupon storage temperature (Table 3).

4. Discussion

Microbial food contamination poses a serious risk to the consumer. *L. monocytogenes* is one of the most important food-borne pathogens. Dairy products are one of the most frequently implicated food in human listeriosis. Milk and dairy products are food with a high nutrient content, willingly consumed by consumers but at the same time enabling good microbial growth. There is a lot of data on the contamination of milk and cheese with *Listeria* spp. (Rahimi et al., 2010). The own study showed the contamination of 10.4% samples of blue cheese with *Listeria* spp. The majority of isolated *Listeria* spp. belonged to the *L. monocytogenes* (6.0%) species, followed by the *L. innocua* (2.8%) and *L. welshimeri* (1.6%) species. Rahimi et al. (2010) found that 17 (28.3%) of the 60 samples of cheese made from raw milk were contaminated with *Listeria* spp., of which *L. monocytogenes* and *L. innocua* were detected in 9 (53%) and 8 (47%) samples, respectively. In the present study *Listeria* spp. were most frequently isolated from Roquefort cheese. Cordano and Rocourt (2001) found no *Listeria* spp. strains in hard cheese samples (155), and the level of soft cheese contamination was assessed at 0.8%. Rudolf and Scherer (2001) showed a higher percentage of *L. monocytogenes* contamination of soft and semi-soft cheese from pasteurized milk (8.0%) than in cheese made from raw milk (4.8%). In turn, Lahou and Uyttendaele (2017) found the presence of *L. monocytogenes* among 3.1% of the 32 soft cheese samples in Belgium, and Lambertz et al. (2012) showed that in Sweden only 0.4% of the 525 samples were contaminated with *L. monocytogenes*. In own study, the highest (14%) degree of contamination was demonstrated for Roquefort cheese. Camembert is the cheese most frequently tested for the presence of *L. monocytogenes* (Linton et al., 2008; Kapetanakou et al., 2017). In the present study, the degree of contamination of Camembert *L. monocytogenes* was found at 6%, whereas in the case of Bri cheese - 4%. Prencipe et al. (2010) showed that 1.0% of the 300 Bri cheese samples in Italy were contaminated with *L. monocytogenes*, and they did not

Table 3
Parameters describing the survival of *Listeria* spp. in a biofilm on stainless steel, depending on the temperature.

Species	Strain	Regression line equation	Theoretical survival time (TST) [day]	Elimination rate (ER) [log CFU × day^{-1}]
Temperature 4 °C				
L. monocytogenes	LMO-B2	y = −0.0623x + 6.4894	104.16^{a}	0.06^{a}
	LMO-C1	y = −0.0581x + 6.5412	112.59^{a,b}	0.06^{a}
	LMO-M1	y = −0.0612x + 6.2086	101.45^{a}	0.06^{a}
	LMO-R3	y = −0.0535x + 6.6557	124.41^{b}	0.05^{a}
L. innocua	LIN-C1	y = −0.0902x + 5.6983	63.17^{c}	0.09^{a,b}
	LIN-G2	y = −0.0948x + 5.6146	59.23^{c}	0.09^{a,b}
	LIN-M1	y = −0.0870x + 5.4219	62.32^{c}	0.09^{a,b}
	LIN-R1	y = −0.0899x + 5.8890	65.51^{c}	0.09^{a,b}
L. welshimeri	LWE-C2	y = −0.0970x + 4.6155	47.58^{d,e}	0.10^{a,b}
	LWE-R1	y = −0.1308x + 6.3758	48.74^{d,e}	0.13^{b,c}
Temperature 20 °C				
L. monocytogenes	LMO-B2	y = −0.1219x + 6.8436	56.14^{c,d}	0.12^{b,c}
	LMO-C1	y = −0.1054x + 6.4759	61.44^{c,d}	0.11^{b}
	LMO-M1	y = −0.1304x + 6.5620	50.32^{c,d}	0.13^{b,c}
	LMO-R3	y = −0.1141x + 7.3869	64.74^{c}	0.11^{b}
L. innocua	LIN-C1	y = −0.1402x + 5.5957	39.91^{e}	0.14^{b,c}
	LIN-G2	y = −0.1359x + 4.7163	34.70^{e}	0.14^{b,c}
	LIN-M1	y = −0.1340x + 5.2215	38.97^{e}	0.13^{b,c}
	LIN-R1	y = −0.1280x + 5.6230	43.93^{d,e}	0.13^{b,c}
L. welshimeri	LWE-C2	y = −0.1619x + 4.7832	29.54^{e,f}	0.16^{c,d}
	LWE-R1	y = −0.1722x + 5.7781	33.55^{e}	0.17^{c,d}
Temperature 37 °C				
L. monocytogenes	LMO-B2	y = −0.2067x + 5.8981	28.53^{e,f}	0.21^{d,f}
	LMO-C1	y = −0.2092x + 6.1834	29.56^{e,f}	0.21^{d,f}
	LMO-M1	y = −0.2025x + 5.7434	28.36^{e,f}	0.20^{d,f}
	LMO-R3	y = −0.1944x + 6.3187	32.50^{e}	0.19^{d}
L. innocua	LIN-C1	y = −0.3378x + 5.0441	14.93^{f}	0.34^{e}
	LIN-G2	y = −0.3610x + 5.0008	13.85^{f}	0.36^{e}
	LIN-M1	y = −0.3347x + 4.7528	14.20^{f}	0.33^{e}
	LIN-R1	y = −0.2534x + 4.7852	18.88^{f}	0.25^{f}
L. welshimeri	LWE-C2	y = −0.3458x + 4.8375	13.99^{f}	0.35^{e}
	LWE-R1	y = −0.3638x + 5.2536	14.44^{f}	0.36^{e}

a, b, c, … - values marked with different letters differ significantly.
Regression line equation: y = ax + b; TST = b/a; ER = a.

detect the presence of these bacteria among 178 samples of Camembert cheese. However, they showed the presence of *L. monocytogenes* among 4.7% of the 444 samples of Gorgonzola cheese (Prencipe et al., 2010). The contamination of 2.1% Gorgonzola samples with *L. monocytogenes* was also demonstrated by Manfreda et al. (2005).

The increasing resistance of *L. monocytogenes* to antibiotics may pose a potential risk to public health. In the present study, it was reported that *L. innocua* and *L. welshimeri* strains were sensitive to all antibiotics tested. Aras and Ardıç (2015) found resistance to ampicillin among nine (56.2%) *L. innocua* strains and three (75%) *L. welshimeri* isolates. Additionally, they detected resistance to penicillin G in two *L. welshimeri* isolates (Aras and Ardıç, 2015). On the other hand, Korsak and Szuplewska (2016) reported that all strains tested of *L. innocua*, *L. welshimeri*, *L. seeligeri* and *L. grayi* were sensitive to ampicillin, ciprofloxacin, erythromycin, gentamycin, rifampicin, trimethoprotein and vancomycin, while two isolates displayed resistance to tetracycline and minocycline. In the present study two (16.7%) *L. monocytogenes* strains were resistant to penicillin and one (8.3%) to erythromycin. Aras and Ardıç (2015) found a greater level of multidrug resistance in *L. monocytogenes* strains (66.7%) than in *L. innocua* (62.5%) and *L. grayi* (53.3%). In contrast, Karadal and Yıldırım (2014) and Conter et al. (2009) did not report any penicillin G-resistant *L. monocytogenes* strains. No ampicillin, meropenem or cotrimoxazole resistance was found in the present study. On the other hand, Ayaz and Erol (2010) and Yücel et al. (2005) recorded resistance to ampicillin among *L. monocytogenes* strains at the 66% and 67.9% level, respectively. Aksoy et al. (2018) found 15 strains of *L. monocytogenes* sensitive to ampicillin and erythromycin, 14 meropenem-sensitive strains and one penicillin-resistant strain. Jamali et al. (2015) and Usman et al. (2016) reported resistance to cotrimoxazole among the *L. monocytogenes* strains tested. In contrast, Mackiw et al. (2016) did not find any cotrimoxazole-resistant strains.

The key factor helping *L. monocytogenes* to survive long periods under adverse environmental conditions is a biofilm production. Ability to create biofilm is an important aspect leading to the secondary food contamination (Doijad et al., 2015). Bacteria may form biofilm in filling machines, on the sides of the gaskets and inside the pipes contributing to the contamination of pasteurized dairy products (Dogan and Boor, 2003; Austin and Bergeron, 1995). Equipment, design and selection of surface materials are important in the prevention of biofilm formation. The most commonly used in the food industry material, used for production surfaces, is stainless steel that can be subjected to mechanical grinding, brushing, electrolytic or mechanical polishing (Maukonen et al., 2003). Although the food processing environment and equipment are regularly cleaned and disinfected, the maintenance of a specific *L. monocytogenes* pulsotype has been documented for various periods of several months to more than ten years (Almeida et al., 2013). In our study, it was shown that three isolates of *L. monocytogenes* from Roquefort and two isolates from Munster were genetically identical. The tested isolates came from various cheese samples from different production periods, which may potentially indicate contamination of food pellets with strains from the production environment.

The survival time of microorganisms in the biofilm structure seems to be a key issue (Doijad et al., 2015). In the present study, *L. monocytogenes* formed the most intensively biofilm on stainless steel whereas *L. welshimeri* was the weakest producer. The number of culturable cells recovered from the biofilm on stainless steel was 6.81 log CFU × cm^{-2} and 4.93 log CFU × cm^{-2} for *L. monocytogenes* and *L. welshimeri*, respectively. This is in accordance with a study of Doijad et al. (2015) who stated that *L. monocytogenes* strains isolated from milk and dairy products produced biofilm the most intensively. The biofilm was

formed on all surfaces tested, used in the industry. They suggested, that unfavorable conditions during milk processing may predispose to more intense biofilm formation. They found no relation between the serotype and the biofilm formation ability (Doijad et al., 2015). On the other hand Osman et al. (2016) reported that not only *L. monocytogenes* strains but also *L. innocua*, *L. welshimeri*, *L. seeligeri* and *L. grayi* strains created a strong biofilm on polystyrene The same affinity for the glass surface was found only in two species (14.3%) (Osman et al., 2016).

We have demonstrated that the weight of dry biofilm biomass varied depending on the species and origin of the strain. A positive correlation was found between the dry weight of the biofilm and the number of culturable cells recovered from it. However, for individual strains with a similar number of culturable cells recovered from the biofilm the dry weight of biofilm was different. This might be explained by the varied composition of extracellular matrix. Sousa et al. (2009) suggested that there is a certain degree of correlation between EPS (Extracellular Polymeric Substances) production and the total amount of biofilm produced. They found that strong biofilm-formers produce higher amounts of EPS, and their amount is strain-dependent (Sousa et al., 2009). In turn, Arslan and Özkardeş (2007) showed that the production of extracellular matrix among most clinical isolates of the central nervous system is related to the ability of these strains to produce more complex biofilms.

The results of the present study revealed that the theoretical survival time of *Listeria* spp. in the biofilm decreased along with the increase of the storage temperature of steel coupons and was strain-dependent. It ranged from 47.58 to 124.41 days at 4 °C, 29.54 to 64.74 days at 20 °C and 13.85 to 32.50 days at 37 °C. This is in agreement with the study of Kadam et al. (2013) who observed that the biofilm production rate increases together with temperature. Takahashi et al. (2011) showed the presence of *L. monocytogenes* (3 log CFU/coupon) after storage in dehydrated state for 30 days. Its survival was significantly higher compared to *S. aureus* or *S.* Typhimurium (Takahashi et al., 2011). Oulahal et al. (2008) showed that *L. innocua* attached to stainless steel in cheese curd reached approximately 3.6 ± 0.2 log CFU × cm^{-2} at 12 °C but was not detected at 25 °C after 8 days. On the other hand, In Lee et al. (2017) demonstrated the ability of all tested *Listeria* spp. strains to create a biofilm on polystyrene microplates and additionally in case of 21 (24.7%) strains, also on stainless steel (at 35 ± 0.5 °C for 48 h). It was emphasized that the strong biofilm formation capacity is not a key factor affecting its durability in the cheese processing environment (In Lee et al., 2017). In the present study, differences in the survival between the tested *Listeria* spp. strains were significant at 4 °C and 20 °C. Oliveira et al. (2010) reported that the number of adherent *L. monocytogenes* cells on the stainless steel surface remained constant after 48 to 192-hour incubation at 37 °C. They also found that the incubation temperature should be consistent with the natural environment from which the strains were isolated (Oliveira et al., 2010).

During storage, transport and retail trade microorganisms might be transmitted from the surfaces to the food (Pérez-Rodrígues et al., 2008). The results of Rodriguez et al. (2008) suggest that stainless steel surfaces transfer more *L. monocytogenes* to food than polyethylene. They showed that as the biofilm dries on stainless steel, the interaction between the cells in the biofilm and between them and the surface weakens. As a result cells are transferred from mature, dried biofilms to moist food products due to capillary effect or water bridge formation (Rodriguez et al., 2008). Therefore, it is extremely important to know the maximum time that cells stay alive in a biofilm in various thermal and humid conditions.

In conclusion, *L. monocytogenes* is the most prevalent species of *Listeria* genus in the mold chess. The ability to form biofilm on the surfaces used in processing plants, including stainless steel, and the difficulty in its elimination increases chance of the secondary contamination of food products posing risk to the consumer health. Therefore, there is a need for further research on the occurrence of *Listeria* spp. in raw material (milk), other types of cheese and in cheese production plants as well as biofilm formation and its survival in a biofilm formed on different materials.

Conflict of interest

Authors declare no conflict of interest.

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