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Elimination of Bacteria *Listeria monocytogenes* in Sewage from Meat Industry in Varied Temperature Conditions

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1. Introduction

Sewage from the meat processing industry poses a potential risk to the water environment, due to the presence of many pathogenic microorganisms, including *Listeria monocytogenes*. The presence of listeria in post-production sewage results from favorable biocenotic conditions that occur during processing of products of animal origin. According to Gill & Jones (1995) and Giovannacci et al. (1999), *L. monocytogenes* can be identified in slaughterhouses even after a year, in spite of washing and disinfections carried out in the processing plant, which results in frequent contamination of meat products (Autio et al., 2000). Moreover, it was proved that there could be more *L. monocytogenes* bacilli in fresh meat than in samples derived from slaughter (Iida et al. 1998). *L. monocytogenes* have been mostly isolated from the surfaces of such devices as: cutters, metal tables, transmission belts, knives and containers for meat (Salvat et al. 1995). Those bacteria can form a biofilm on devices made of stainless steel or aluminum, which increases their ability to survive, especially at temperatures close to 4°C. Somers & Wong (2004) indicated that the presence of meat remnants makes that biological membrane where *L. monocytogenes* bacilli occur more resistant to the processes of decontamination. Biological characteristics of *L. monocytogenes*, including their resistance to the environmental factors, allow them to growth in

the infected organism, as well as in the secondary source of infection (Gliński & Kostro 2012). These bacteria retain their virulence in the wide range of temperatures from -2 to 45°C (Walczycka 2005). Moreover, they are able to survive freezing and short-duration pasteurization (Szosland-Fałtyn et al. 2012). Contamination of sewage with those bacteria is high, which is indicated by the percentage of positive samples reaching up to 93% (Geuenich et al. 1985).

Therefore, a study was carried out which aimed to estimate the elimination rate of three chosen strains of *Listeria monocytogenes* in post-production sewage from meat processing at varied temperatures.

2. Material and methods

Bacteria strains and preparation of inoculum

In this experiment we used three standard strains of *Listeria monocytogenes:* ATCC 19111, ATCC 19114 and ATCC 19115 (Merck). At the first stage, pure cultures of *Listeria monocytogenes* were grown on the agar medium TSA – Trypticasein Soy LAB-AGAR (Merck). After 24 hours of incubation at 37°C grown colonies were transferred to ampules containing 2 ml 0.85% NaCl each, with an addition of 1 g peptone per liter. The number of bacterial cells was determined using the VITEK Systems ATB 1550 densitometer (we estimated the number of bacterial cells present in the prepared suspension at 10^7-10^8 cfu).

The course of the experiment and inoculation of sewage

This experiment was carried out using sewage derived from the meat processing plant located in the Kuyavian-Pomeranian voivodeship. Freshly collected samples of sewage from the meat processing plant were introduced to six sterile containers (two for each strain of *L. monocytogenes*) with a volume of 5000 ml. Then suspensions containing strains of *Listeria monocytogenes* were added in an amount of 10 ml per each 1000 ml of sewage, and then mixed thoroughly and left at the room temperature for 1 hour. Afterwards, the number of cells of individual *Listeria monocytogenes* strains was determined in 1 ml of inoculated sewage. To determine the effect of varied temperatures on the survival rate of *L. monocytogenes*, containers with the studied sewage were stored at two separate places with temperatures of 4 and 20°C.

Microbiological analyses

The experiment was conducted using the MPN (Most Probable Number) method with a 3-tube kit, according to the standard EN ISO 11290-2:1998/A1:2004. At the first stage of the study, the liquid half-Fraser medium (Merck) was used, from which a series of decimal solutions (10^{-1}-10^{-9}) of inoculated sewage were made. After incubation at 30°C after 24 hours, from the cultures of positive and dubious cases inoculations were made on selective media: PALCAM agar (Merck), Ottaviani and Agostini (ALOA; Merck) and Oxford-agar (Merck). Incubation was carried out at 37°C for 24 hours. After that definite incubation time, the obtained results were interpreted and the colonies were identified. Typical colonies of *L. monocytogenes* on the ALOA medium are green-blue and they were surrounded by the opaque zone of the medium. On the Oxford-agar medium, typical colonies of *L. monocytogenes* are dark gray with a greenish tone, and a change of the medium color into black is observed. Typical colonies of *L. monocytogenes* on the PALCAM agar medium are grey with a green tone, and the medium also changes color into black. To confirm the presence of *Listeria monocytogenes*, biochemical tests API Listeria were performed, as well as Gram staining, tests for the type of hemolysis and for catalase.

Statistical analysis

Results of the tests for survival of *Listeria monocytogenes* were verified and subjected to the statistical analysis based on changes in the number of bacteria in time, according to the formula:

$$\log(N) = ax + b \quad (1)$$

where: N – the number of bacteria present at the definite time in the studied sewage, a – directional index corresponding to the average change in the number of bacteria (log cfu), x – time [week], b – free expression corresponding to the logarithm of the number of *Listeria monocytogenes* in time 0.

The results were subjected to logarithmic transformation and then regression lines were drawn using the software Microsoft Office Excel 2007.

3. Results and discussion

The results of the present study concerning the survival of *Listeria monocytogenes* in sewage from meat industry in varied temperature conditions are presented in Tables 1-3 and in Figures 1-6. At the beginning of the experiment, in sewage samples stored at 4°C the number of cells of *L. monocytogenes* ATCC 19111 amounted to $1.5 \cdot 10^8$ cfu/ml, the number of ATCC 19114 was $2.5 \cdot 10^8$ cfu/ml, whereas that of ATCC 19115 – $7.5 \cdot 10^7$ cfu/ml (Tab. 1). It is notable that in the strain ATCC 19114, a slight increase in the number of those bacteria was observed in the second week of the study. Kołakowska & Madajczak (2011) confirm the ability of these microorganisms to multiply at low temperatures.

Table 1. Number of *L.monocytogenes* in sewage at 4°C
Tabela 1. Liczba *L.monocytogenes* w ściekach w 4°C

Weeks	*Listeria monocytogenes* ATCC 19111	*Listeria monocytogenes* ATCC 19114	*Listeria monocytogenes* ATCC 19115
	cfu/ml	cfu/ml	cfu/ml
1	$1.5 \cdot 10^8$	$2.5 \cdot 10^8$	$7.5 \cdot 10^7$
2	$9.5 \cdot 10^7$	$3.5 \cdot 10^8$	$2.5 \cdot 10^7$
3	$4.5 \cdot 10^7$	$3.0 \cdot 10^7$	$9.5 \cdot 10^6$
4	$2.5 \cdot 10^7$	$2.5 \cdot 10^7$	$7.5 \cdot 10^5$
5	$2.5 \cdot 10^6$	$9.5 \cdot 10^6$	$3.0 \cdot 10^5$
6	$9.5 \cdot 10^3$	$9.5 \cdot 10^4$	$9.5 \cdot 10^2$
7	$2.5 \cdot 10^3$	$2.5 \cdot 10^4$	$2.5 \cdot 10^2$
8	$7.5 \cdot 10^2$	$1.5 \cdot 10^4$	$1.5 \cdot 10^2$
9	$2.5 \cdot 10^2$	$7.5 \cdot 10^3$	$9.5 \cdot 10^1$
10	$2.5 \cdot 10^1$	$3.0 \cdot 10^3$	$4.5 \cdot 10^1$
11	$1.5 \cdot 10^1$	$9.5 \cdot 10^1$	$2.5 \cdot 10^1$
12	0.0	$4.5 \cdot 10^1$	0.0
13	0.0	$1.5 \cdot 10^1$	0.0
14	0.0	0.0	0.0

In the initial period of the present study, i.e. for the first 4 weeks, a slight decrease in the number of cells of *L. monocytogenes* by one logarithmic unit was observed in the first two strains, whereas by two units in the strain ATCC 19115. In the 11^{th} week of the study there was a marked

decrease in the number of indicator bacteria to a level of 10^1 cfu in 1 ml of sewage. In the next week of the experiment, no cells of *L. monocytogenes* ATCC 19111 or ATCC 19115 were recorded in the sewage, whereas bacteria of the strain ATCC 19114 were inactivated only in the 14th week of the study. The long survival period of *L. monocytogenes* ATCC 19114 in sewage derived from the meat industry at 4°C was also observed by Budzińska et al. (2012), since on the 100th day of the study the number of those bacteria was at a level of $4.8 \cdot 10^2$ cfu/ml. Rysert & Marth (2007) also confirm a high adaptation of *L. monocytogenes* to low temperatures. Undoubtedly, this phenomenon can be explained with the psychotropic nature of the studied bacteria (Fiedoruk & Zaremba 2010). Hansen et al. (2006) report that those bacteria survived definitely shorter in the water environment at 5°C, maximally until the 40th day of the experiment.

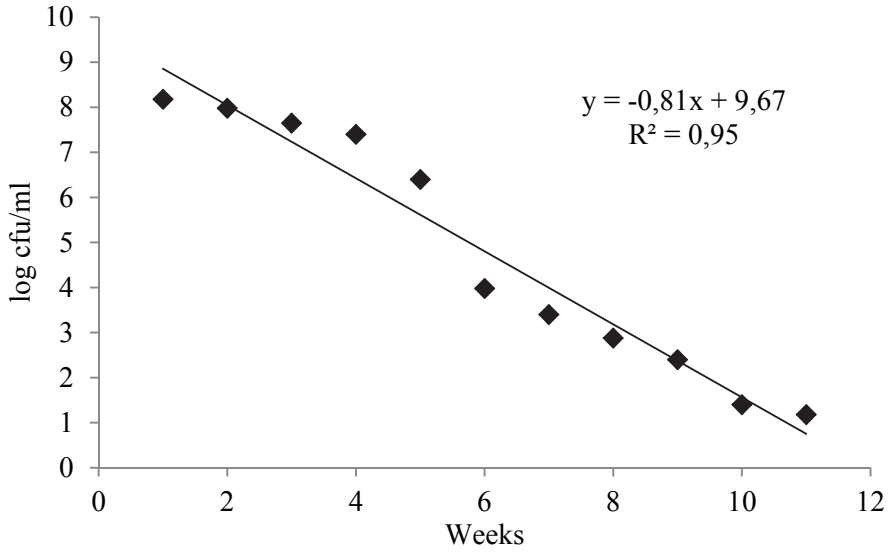

Fig. 1. Survival of *L. monocytogenes* ATCC 19111 in sewage at 4°C
Rys. 1. Przeżywalność *L. monocytogenes* ATCC 19111 w ściekach 4°C

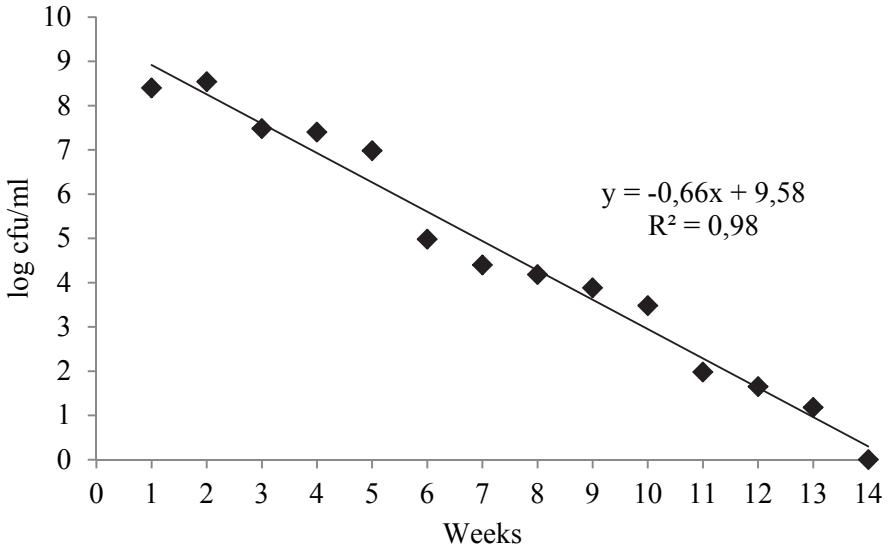

Fig. 2. Survival of *L. monocytogenes* ATCC 19114 in sewage at 4°C
Rys. 2. Przeżywalność *L. monocytogenes* ATCC 19114 w ściekach w 4°C

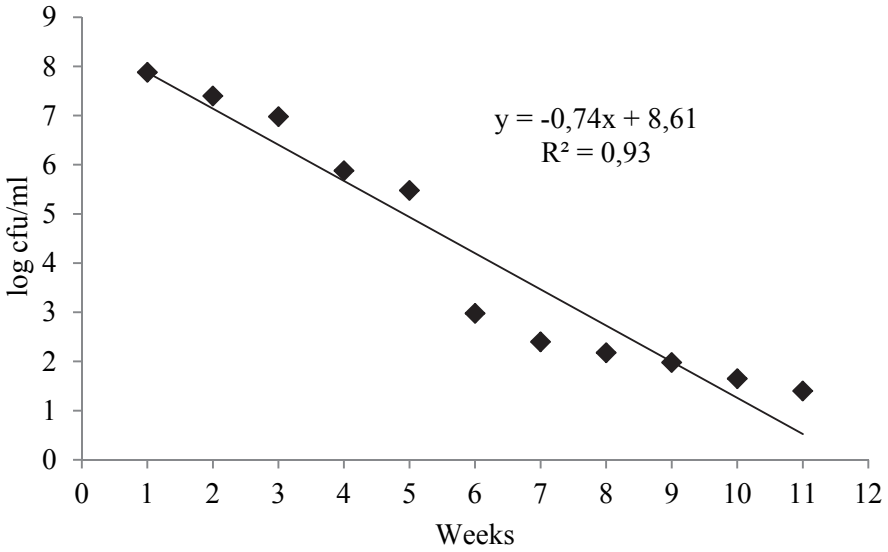

Fig. 3. Survival of *L. monocytogenes* ATCC 19115 in sewage at 4°C
Rys. 3. Przeżywalność *L. monocytogenes* ATCC 19115 w ściekach w 4°C

In the present study, at 4°C it was indicated that the weekly elimination rate of bacteria *L. monocytogenes* derived from sewage ranged from 0.66 (ATCC 19114) to 0.81 (ATCC 19111) log cfu/ml. Cells of the strain ATCC 19115 died at a rate of 0.74 log cfu/week (Fig. 1-3, Tab. 3). In the study conducted by Budzińska et al. (2012), the elimination rate of *L. monocytogenes* ATCC 19114 in sewage amounted to 0.06 log cfu/day. Besnard et al. (2002) indicate that at 4°C in the environment of water and sewage *Listeria monocytogene*s turn to the VBNC (viable but non-culturable) stage, i.e. cells that are live but not cultured on media. This phenomenon occurs in different environmental stress conditions, whereas the bacteria retain their pathogenicity and have active metabolism.

In sewage stored at 20°C the initial number of cells of individual *L.monocytogenes* strains was at the same level as in sewage stored at 4°C. After the first week of the experiment no rapid decrease was recorded in the number of the studied bacteria, and it ranged from $7.5 \cdot 10^7$ to $2.5 \cdot 10^8$ cfu/ml (Tab. 2). In the strain ATCC 9111, after 4 weeks of the experiment there was a decrease in the number of bacteria by 4 logarithmic units, whereas in ATCC 19115 by as many as 5 logarithmic units. Cells of the strain ATCC 19114 were isolated from sewage at 20°C for the longest time, since in the 5^{th} week of the study they still had a level of $1.5 \cdot 10^1$ cfu/ml, whereas the other two strains underwent inactivation earlier. Statistical calculations (Fig. 4-5, Tab. 3) showed that weekly elimination rate of the tested bacteria at 20°C was different for individual strains and amounted to 2.03 log cfu (ATCC 19111), 1.86 log cfu (ATCC 19114) and 1.75 log cfu (ATCC 19115), respectively. Obtained results of the present study proved that during the experiment all the tested strains of *L. monocytogenes* survived shorter at 20°C than at 4°C. A similar tendency to inactivation of *L. monocytogenes* cells in the water environment was noted by Hansen et al. (2006). In the experiment conducted by Budzińska et al. (2012), daily elimination rate of *L. monocytogenes* ATCC 19114 was at a level of 0.14 log cfu. The authors also confirmed that the elimination rate of those microorganisms proceeded faster at 20°C. However, in other experiments conducted by Budzińska & Wroński (2008), the elimination rate of cells of this strain in sewage was higher and amounted to 0.23 log cfu.

In the present study (Tab. 3) the theoretical maximal survival time of *L. monocytogenes* at 4°C calculated from regression equations was the

longest for the strain ATCC 19114 and amounted to 14.50 weeks (102 days), whereas cells of the two other strains, ATCC 19111 and ATCC 19115, survived definitely shorter, from 11.90 weeks (84 days) and 11.60 weeks (82 days).

Table 2. Numbers of *L.monocytogenes* in sewage at 20°C
Tabela 2. Liczba *L. monocytogenes* w ściekach w 20°C

Weeks	*Listeria monocytogenes* ATCC 19111	*Listeria monocytogenes* ATCC 19114	*Listeria monocytogenes* ATCC 19115
	cfu/ml	cfu/ml	cfu/ml
1	$1.5 \cdot 10^8$	$2.5 \cdot 10^8$	$7.5 \cdot 10^7$
2	$7.5 \cdot 10^7$	$1.5 \cdot 10^6$	$2.5 \cdot 10^5$
3	$1.5 \cdot 10^4$	$2.5 \cdot 10^4$	$4.5 \cdot 10^2$
4	$4.5 \cdot 10^2$	$9.5 \cdot 10^1$	$1.0 \cdot 10^1$
5	0.0	$1.5 \cdot 10^1$	0.0
6	0.0	0.0	0.0

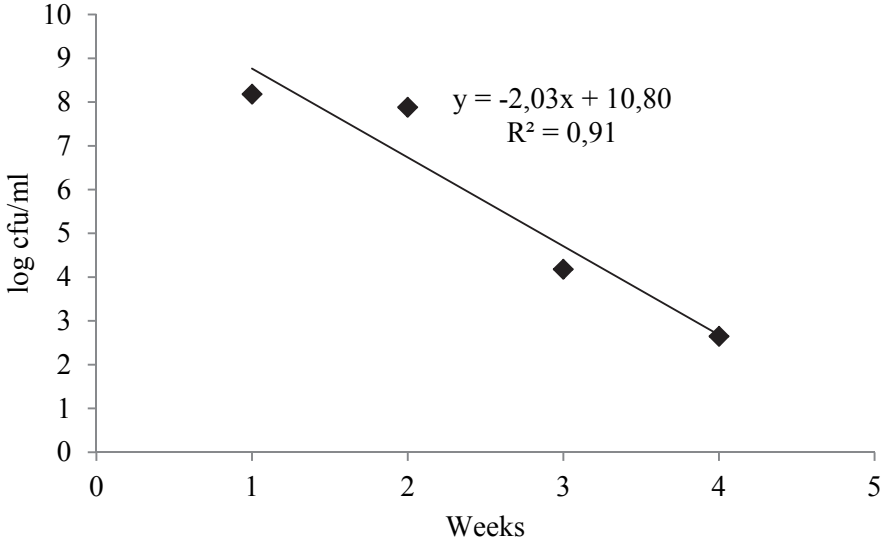

Fig. 4. Survival of *L. monocytogenes* ATCC 19111 in sewage at 20°C
Rys. 4. Przeżywalność *L. monocytogenes* ATCC 19111 w ściekach w 20°C

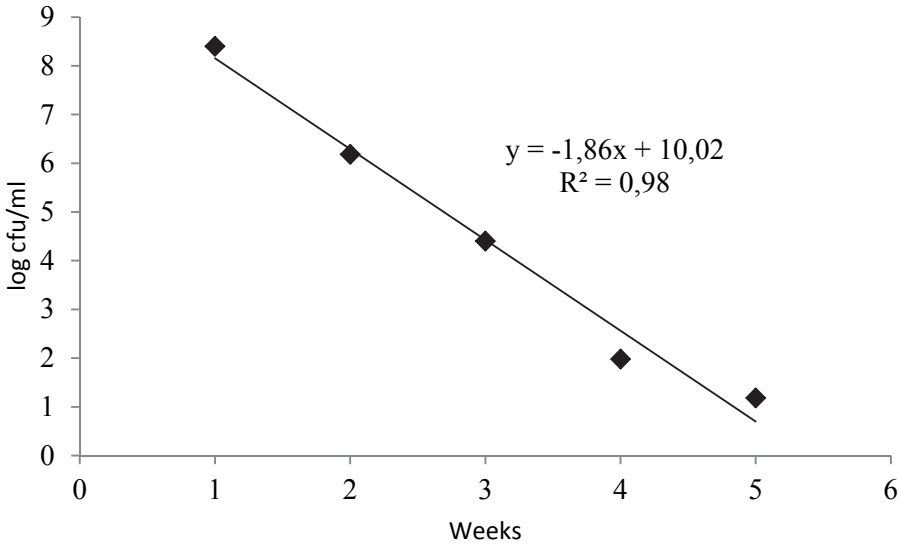

Fig. 5. Survival of *L. monocytogenes* ATCC 19114 in sewage at 20°C
Rys. 5. Przeżywalność *L. monocytogenes* ATCC 19114 w ściekach w 20°C

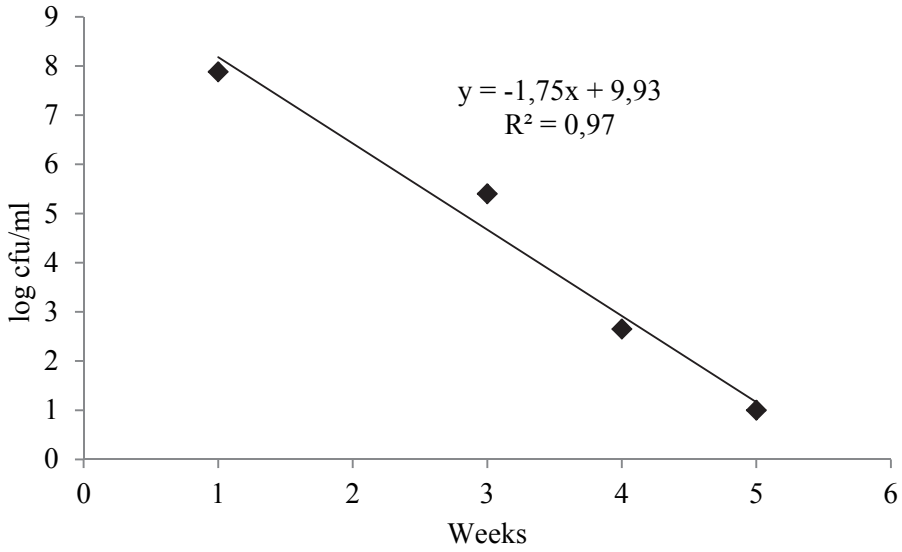

Fig. 6. Survival of *L. monocytogenes* ATCC 19115 in sewage at 20°C
Rys. 6. Przeżywalność *L. monocytogenes* ATCC 19115 w ściekach w 20°C

At 20°C, the survival time of the tested strains was similar and ranged from 5.3 weeks (37 days) to 5.7 weeks (40 days). Similar results were noted by Budzińska & Wroński (2008), where the theoretical survival time of *L. monocytogenes* ATCC 19114 in raw sewage amounted to 38 days at their initial number of $9.5 \cdot 10^9$ cfu/ml.

Table 3. Elimination rate and maximal survival time of *L. monocytogenes* in sewage
Tabela 3. Tempo eliminacji oraz maksymalny czas przeżycia pałeczek *L. monocytogenes* w ściekach

Specification	Temperature 4°C		Temperature 20°C	
	Weekly elimination rate (log cfu)	Theoretical maximal survival time (week)	Weekly elimination rate (log cfu)	Theoretical maximal survival time (week)
L. monocytogenes ATCC 19111	0.81	11.90	2.03	5.30
L. monocytogenes ATCC 19114	0.66	14.50	1.86	5.40
L. monocytogenes ATCC 19115	0.74	11.60	1.75	5.70

Sewage contaminated with *L. monocytogenes* bacilli performs the crucial role in transmission of those bacteria in the water environment, which consequently may be the cause of their presence in surface waters (Czeszejko et al. 2003). *L. monocytogenes* can survive in the water environment even for 300 days, not losing their virulence (Dykes & Moorhead 2001, Ryser & Marth 2007), therefore they constitute the etiological factor of listeriosis, a disease that is dangerous for both people and animals (Arvanitidou et al. 1997, Lyautey et al. 2007).

4. Conclusions

1. *Listeria monocytogenes* underwent gradual elimination in sewage derived from the meat processing plant, whereas the elimination rate of cells of those microorganisms depended on the temperature conditions and the tested strain.

2. The weekly elimination rate of *L. monocytogenes* at 4°C was 0.81 log cfu for the strain ATTC 19111, 0.66 log cfu for ATTC 19114 and 0.74 log cfu for ATTC 19115. Definitely faster inactivation rate of those strains was observed at 20°C and it amounted to: 2.03; 1.86 and 1.75 log cfu, respectively.
3. A longer survival time of all the studied strains of *L. monocytgenes* in sewage was recorded at 4°C than at 20°C. The strain ATTC 19114 survived longer by 64 days at the lower temperature, whereas the strains ATTC 19111 and ATTC 19115, by 47 and 42 days, respectively.
4. The present study indicates the need for monitoring of sewage disposed from the meat industry plants for microbiological pollution of surface waters.

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Eliminacja bakterii *Listeria monocytogenes* w ściekach z przemysłu mięsnego w zróżnicowanych warunkach temperaturowych

Streszczenie

Ścieki z zakładów przetwórstwa mięsnego stanowią potencjalne zagrożenie dla środowiska wodnego z powodu obecności w nich wielu drobnoustrojów patogennych, w tym pałeczek *Listeria monocytogenes*. Kontaminacja tymi bakteriami ścieków nieoczyszczonych jest wysoka o czym świadczy odsetek pozytywnych próbek osiągający poziom do 93%. Celem przeprowadzonych badań było ustalenie tempa eliminacji trzech wybranych szczepów *Listeria monocytogenes* ATCC 19111; ATCC 19114 oraz ATCC 19115 w ściekach poprodukcyjnych z przetwórstwa mięsnego w temperaturze 4 i 20°C. Badania przeprowadzono zgodnie z normą EN ISO 11290-2:1998/A1:2004. Na początku eksperymentu w próbkach ścieków przechowywanych w temperaturze 4°C liczba komórek *L. monocytogenes* ATCC 19111 wynosiła $1{,}5 \cdot 10^8$ jtk/ml, ATCC 19114 przyjmowała wartość $2{,}5 \cdot 10^8$ cfu/ml, natomiast ATCC 19115 – $7{,}5 \cdot 10^7$ jtk/ml. W początkowym okresie badań przez pierwsze 4 tygodnie odnotowano nieznaczny spadek liczby komórek *L. monocytogenes* w przypadku dwóch pierwszych szczepów o jedną jednostkę logarytmiczną, natomiast w przypadku szczepu ATCC 19115 o dwie jednostki. W 11 tygodniu badań nastąpiło zdecydowane obniżenie liczby bakterii wskaźnikowych do poziomu 10^1jtk w 1 ml ścieków. W kolejnym tygodniu eksperymentu nie odnotowano w ściekach obecności komórek *L. monocytogenes* ATCC 19111 i ATCC 19115, natomiast bakterie szczepu ATCC 19114 uległy inaktywacji dopiero w 14 tygodniu badań. W temperaturze 4°C wykazano tygodniowe tempo eliminacji bakterii *L. monocytogenes* ze ścieków od 0,66 (ATCC 19115) do 0,81 (ATCC 19111) log jtk/ml. Z kolei komórki szczepu ATCC 19114 obumierały w tempie

0,74 log jtk/tydzień. W ściekach przechowywanych w temperaturze 20°C początkowa liczba komórek poszczególnych szczepów *L.monocytogenes* kształtowała się na takim samym poziomie, jak w ściekach w temperaturze 4°C. W przypadku szczepu ATCC 9111 po 4 tygodniach doświadczenia nastąpiło obniżenie liczby bakterii o 4 jednostki logarytmiczne, natomiast w przypadku ATCC 19115 aż o 5 jednostek logarytmicznych. Najdłużej izolowano ze ścieków w temperaturze 20°C komórki szczepu ATCC 19114, ponieważ w 5 tygodniu badań występowały one jeszcze na poziomie $1{,}5 \cdot 10^1$ jtk/ml, gdy tymczasem dwa pozostałe szczepy uległy wcześniej inaktywacji. Obliczenia statystyczne wykazały, że tygodniowe tempo eliminacji badanych bakterii w temperaturze 20°C było zróżnicowane dla poszczególnych szczepów i wynosiło odpowiednio 2,03 log jtk (ATCC 19111), 1,86 log jtk (ATCC 19114) i 1,75 log jtk (ATCC 19115). Uzyskane wyniki badań dowiodły, że w trakcie doświadczenia wszystkie testowane szczepy *L. monocytogenes* przeżywały krócej w temperaturze 20°C niż w temperaturze 4°C. Teoretyczny maksymalny czas przeżycia pałeczek *L. monocytogenes* w temperaturze 4°C był najdłuższy dla szczepu ATCC 19114 i wynosił 14,5 tygodni (102 dni), natomiast komórki dwóch pozostałych szczepów ATCC 19111 i ATCC 19115 przeżywały zdecydowanie krócej od 11,9 tygodnia (84 dni) i 11,6 tygodnia (82 dni). Z kolei w temperaturze 20°C czas przeżycia testowanych szczepów był podobny i wynosił od 5,3 tygodni (37 dni) do 5,7 tygodni (40 dni).

Abstract

Sewage from the meat processing industry present a potential risk to the water environment, due to the presence of many pathogenic microorganisms, including *Listeria monocytogenes*. Contamination of untreated sewage with those bacteria is high, which is expressed by the percentage of positive samples reaching up to 93%. The aim of the study was to determine the elimination rate of three chosen strains of *Listeria monocytogenes* ATCC 19111; ATCC 19114 and ATCC 19115 in meat processing post-production sewage at temperatures 4 and 20°C. Experiments were conducted according to the standard of EN ISO 11290-2:1998/A1:2004. At the beginning of the experiment, in sewage samples stored at 4°C the number of cells of *L. monocytogenes* ATCC 19111was amounted to $1.5 \cdot 10^8$ cfu/ml, the number of ATCC 19114 was $2.5 \cdot 10^8$ cfu/ml, however the number of ATCC 19115 was $7.5 \cdot 10^7$ cfu/ml. In the initial period of the study, i.e. for the first 4 weeks, a slight decrease by one logarithmic unit of the *L. monocytogenes* cells number was observed in the first two strains, whereas of the strain ATCC 19115 by two units. In the 11^{th} week of the study there was a marked decrease in the number of indicator bacteria to a level of 10^1 cfu in 1 ml of sewage. In the next week of the experiment, no cells of *L. monocyto-*

genes ATCC 19111 or ATCC 19115 were noticed in the sewage, whereas bacteria of the strain ATCC 19114 were inactivated first in the 14^{th} week of the study. In the temperature of 4°C it was shown, that the weekly elimination rate of bacteria *L. monocytogenes* derived from sewage ranged from 0.66 (ATCC 19114) to 0.81 (ATCC 19111) log cfu/ml. Cells of the strain ATCC 19115 expired at a rate of 0.74 log cfu/week. In sewage stored at 20°C the initial number of cells of individual *L.monocytogenes* strains was at the same level as in sewage stored at 4°C. After the first week of the experiment no rapid decrease was recorded in the number of the studied bacteria, and it ranged from $7.5 \cdot 10^7$ to $2.5 \cdot 10^8$ cfu/ml. In the strain ATCC 9111, after 4 weeks of the experiment there was a decrease in the number of bacteria by 4 logarithmic units, whereas in ATCC 19115 by 5 logarithmic units. Cells of the strain ATCC 19114 were isolated from sewage at 20°C for the longest time, since in the 5^{th} week of the study they still had a level of $1.5 \cdot 10^1$ cfu/ml, whereas the other two strains underwent inactivation earlier. Statistical calculations showed that weekly elimination rate of the tested bacteria at 20°C was different for individual strains and amounted respectively to 2.03 log cfu (ATCC 19111), 1.86 log cfu (ATCC 19114) and 1.75 log cfu (ATCC 19115). Obtained results of the present study proved that during the experiment all the tested strains of *L. monocytogenes* survived shorter at 20°C than at 4°C. In the presented study the theoretical maximal survival time of *L. monocytogenes* at 4°C calculated from regression equations was the longest for the strain ATCC 19114 and amounted to 14.50 weeks (102 days), whereas cells of the two other strains, ATCC 19111 and ATCC 19115, survived definitely shorter, from 11.90 weeks (84 days) and 11.60 weeks (82 days). At 20°C, the survival time of the tested strains was similar and ranged from 5.3 weeks (37 days) to 5.7 weeks (40 days).

Słowa kluczowe:
ścieki, *Listeria monocytogenes*, przeżywalność, temperatura

Keywords:
sewage, *Listeria monocytogenes*, survival, temperature