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Original Research

Survival Time of Bacteria *Listeria monocytogenes* in Water Environment and Sewage

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Abstract

In recent years there has been a growing interest in hazards having a microbiological source, resulting from the direct transmission of microorganisms from the natural environment, which may result in inducing diseases in people and animals. This study aimed at determining the survival of pathogenic bacteria *Listeria monocytogenes* in water and sewage from the meat industry plant, depending on diversified temperature conditions, as well as the assessment of sanitary and epidemiological risks resulting from the ability of determined microorganisms to live in a water environment. On the basis of our research, it was found that *Listeria monocytogenes* showed the longest survival time both in water and sewage at 4ºC, where the maximal survival time of those microorganisms determined on the basis of the regression analysis amounted to 120 and 141 days, respectively. The results obtained in the experiment present important data concerning the survival of the bacteria *Listeria monocytogenes* in sewage and the water environment, and at the same time they draw attention to the need for a constant monitoring of effluents from the meat industry plant, as well as the surface waters within the range of influence of those facilities on pathogenic and potentially pathogenic microorganisms.

Keywords: *Listeria monocytogenes*, temperature, survival time, water, sewage from meat industry plants

Introduction

Listeria monocytogenes is a Gram-positive, facultative intracellular pathogen of people and animals, widely spread throughout land and water environments. It is often isolated from samples of different kinds, such as soil, faeces, decaying plant material, vegetables, and silage [1-4]. A high proportion of the bacteria *Listeria monocytogenes* was observed in treated household and industrial sewage, including effluents deriving from meat industry plants. Contaminated sewage plays an essential role in transmission of listeria in the water environment, which consequently can be a cause of their presence in rivers and lakes, as well as sea and ground waters [5-8]. The literature data determine the frequency of the bacteria *Listeria monocytogenes* occurrence in samples of the tested waters reaching up to 62% [9]. It is also notable that these bacteria, owing to a high resistance to unfavourable external conditions, are able to survive in the environment for a long time [10-12]. *Listeria monocytogenes* is classified as a typical psychrotroph and it can develop within a wide range of temperatures, from -1.5 to 50ºC [13]. Many authors indicate its particular predisposition to growth and multiply at low temperatures, whereas the growth of the majority of other pathogenic microorganisms is stunted [14-16]. Because of this fact, the presence of *Listeria monocytogenes* bacilli in water ecosystems may be a cause of sporadic or epidemic listeriosis incidence, which poses a serious hazard for people and animal health [17-19].

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The first clinically supported case of isolation of the bacteria *Listeria monocytogenes* in people was described in 1929. However, a noticeable growth in incidence caused by these bacteria in people began to be recorded in many countries only after 1980 [12]. In recent years, listeriosis was ranked fifth in respect of the most frequently occurring infections in Europe, after infections with *Campylobacter*, *Salmonella*, *Yersinia*, and *Escherichia coli* O157:H7 [20, 21]. It is estimated that up to 5% or more healthy people are asymptomatic hosts of *Listeria monocytogenes* [3]. Infections with those bacteria are characterized by a high level of mortality and apply mainly to elderly people, pregnant women, infants and immunocompromised individuals. Meningitis, encephalitis, and septicaemia are usually recorded in the course of listeriosis, as well as abortions, premature births, and infant infections [22-25].

The aim of this study was to estimate in model research the time of survival of the bacteria *Listeria monocytogenes* in water and sewage from the meat industry plants, depending on diversified temperature conditions, as well as to assess the sanitary and epidemiological risk resulting from the ability of the tested microorganisms to exist in the water environment.

Experimental Procedures

Bacterial Strain and Inoculum Preparation

The standard strain of *Listeria monocytogenes* ATCC 19114 (Kwik-Stik, MicroBioLogics) was used in the study. According to the growth requirements, it was inoculated on the medium Tryptic Soy Agar (CASO Agar, Merck). After 24 hours of incubation at 37ºC, single colonies were collected from the inoculated medium and in order to prepare the bacteria suspension, they were introduced into an ampoule containing 2 ml of 0.85% NaCl solution with an addition of 1 $g \cdot l^{-1}$ peptone. The concentration of *Listeria monocytogenes* in the prepared suspension remained at a level of 10^9 $CFU \cdot ml^{-1}$. The number of bacteria cells was determined using the densitometer Vitek Systems ATB 1550 (bioMérieux).

Experimental Design and Inoculation

To conduct this experiment, samples of post-production sewage were collected deriving from the meat industry plant and river water within the range of the effect of this facility. After transporting to the laboratory samples of water and sewage were introduced into four sterile glass containers with a volume of 5000 ml (two samples with water and two samples with sewage) and inoculated with the prepared suspension of the bacteria *Listeria monocytogenes* ATCC 19114. After one hour from establishing the experiment, the concentration of bacteria in 1 ml of the tested material (initial sample) was determined. Then inoculated samples were placed in chambers with varied temperature conditions, i.e. at 4 and 20ºC. On appointed days of the experiment, the count of the bacteria *Listeria monocytogenes* was determined in individual samples of water and sewage, based on the most probable number of microorganisms (MPN) in the three-tube design. The experiment was carried out in three replications.

Microbiological Analysis

The assessment of survival of the strain *Listeria monocytogenes* ATCC 19114 in water and sewage was carried out based on the following norms: PN-EN ISO 11290-1:1999/A1:2005 and PN-EN ISO 11290-2:2000/A1:2005 [26, 27], using a modification resulting from the character of the tested samples. Microbiological analyses were made according to the following procedure. The first stage involved initial multiplication in a selective liquid medium with a lowered content of selectively inhibiting factors – that is in the half-Fraser broth (Fraser *Listeria* Selective Enrichment Broth, Merck). In the half-Fraser medium, decimal dilutions of water and sewage from 10^{-1} to 10^{-10} were made, and the samples obtained were incubated at 30ºC for 24 hours. Blackening of the medium occurring after this time indicated a possibility of the occurrence of *Listeria monocytogenes* in the tested samples. At the next stage, from the positive cultures inoculations were made on two selective solid media – inhibiting the growth of commonly occurring Gram-negative bacteria and the most of Gram-positive bacteria – the agar medium *Listeria* according to Ottaviani and Agosti (ALOA) (Chromocult *Listeria* Selective Agar acc. to Ottaviani and Agosti, Merck) and the agar PALCAM (*Listeria* PALCAM Selective Agar acc. to VAN NETTEN et al., Merck). Incubation of inoculated media was carried out at 37ºC for 24 hours, and in doubtful cases (i.e. the lack of growth or a weak growth in the first reading), up to 48 hours. On the agar medium *Listeria* according to Ottaviani and Agosti (ALOA), typical colonies of *Listeria monocytogenes* after 24-48 hours of incubation were of green-blue color, surrounded with a non-transparent zone. On agar PALCAM after 24 hours of incubation typical colonies of the tested bacteria grew in the form of small grey-green or olive-green colonies, from 1.5 mm to 2 mm in diameter, surrounded with a zone of the black-colored medium, sometimes with the black-colored centre, whereas after 48 hours of incubation they occurred in the form of green colonies, with a diameter of 1.5-2 mm with a characteristic hollow in the middle, surrounded with a zone of the black-colored medium. Colonies grown on selective solid media were subject to confirming tests towards *Listeria monocytogenes*. The range of conducted identification involved dyeing with the Gram method, tests for producing catalase, and determining the type of hemolysis, as well as tests for the ability to decompose carbohydrates based on the biochemical system API *Listeria* (bioMérieux).

Statistical Analysis

The results of the research on the survival time of *Listeria monocytogenes* in water and sewage were analyzed statistically on the basis of using the following model of bacteria kinetic inactivation in time:

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

...where:

N – the number of bacteria in the given time in water or sewage

a – the directional coefficient corresponding to the average change in the number of bacteria in the form of log per one day

x – time in days

b – the constant term theoretically corresponding to log of the number of bacteria in the zero time, involved in the given process.

The coefficients of regression and determination, as well as values of standard deviation and error were calculated. The theoretical time of survival and the elimination rate of *Listeria monocytogenes* in water and sewage were determined on the basis of the course of regression lines. The analysis of the results obtained was carried out using the program STATISTICA 8.0.

Results and Discussion

The results of changes in the number of *Listeria monocytogenes* in the water environment and in sewage from the meat industry plant depending on the temperatures of 4 and 20ºC obtained in this study were presented in Tables 1 and 2 and Figs. 1-4.

Water can play an important role in transmission of the bacteria *Listeria monocytogenes*, which causes a disease dangerous for people and animals called listeriosis. Respect for sanitary state, the fact that these bacteria are often isolated from the water environment and, consequently, pose a potential hazard to the public health is of the utmost importance [7, 17]. According to Czeszejko et al. [5] a high degree of contamination of the Odra waters with the bacteria *Listeria monocytogenes* was observed in Poland, which can reach a value of 54.5%. Considerable pollution of the water environment with listeria bacilli is undoubtedly connected with their presence in sewage, including the effluents from meat industry plants. Additionally, the ineffectiveness of mechanical-biological treatment should be noted, as well as the effect of various ranges of the pH values on the effective inactivation of *Listeria monocytogenes* from meat industry sewage, which consequently may be a cause of deterioration in the microbiological state of surface waters [5, 28].

During the author's experiment, a gradual elimination of the *Listeria monocytogenes* bacilli in the water environment was observed, whereas the rate of their inactivation was noticeably dependent on the temperature of the tested sample. At 4ºC, the initial number of *Listeria monocytogenes* in water took a value of $2.5 \cdot 10^8$ CFU·ml^{-1} and decreased on the 10th day of the study to a level of $2.5 \cdot 10^7$ CFU·ml^{-1}. Then on the 20th day of the experiment, listeria bacilli were isolated in a number of $3.0 \cdot 10^6$ CFU·ml^{-1}, which still occurred at a similar level on the 30 and 40th days of the experiment. Then their concentration gradually fell, reaching a value of $1.5 \cdot 10^1$ CFU·ml^{-1} on the 100th day of the experiment (Table 1, Fig. 1).

Table 1. Quantitative changes of *Listeria monocytogenes* in water samples on particular days of research.

Days of experiment	Mean number of *Listeria monocytogenes* [CFU·ml^{-1}] at 4ºC	Days of experiment	Mean number of *Listeria monocytogenes* [CFU·ml^{-1}] at 20ºC
0	$2.5 \cdot 10^8$	0	$9.5 \cdot 10^8$
10	$2.5 \cdot 10^7$	5	$2.5 \cdot 10^8$
20	$3.0 \cdot 10^6$	10	$4.5 \cdot 10^7$
30	$1.0 \cdot 10^6$	15	$2.5 \cdot 10^5$
40	$1.5 \cdot 10^6$	20	$1.6 \cdot 10^5$
50	$2.5 \cdot 10^5$	25	$9.5 \cdot 10^4$
60	$1.0 \cdot 10^5$	30	$1.5 \cdot 10^2$
70	$4.5 \cdot 10^3$	35	$1.0 \cdot 10^2$
80	$2.5 \cdot 10^2$	40	$1.0 \cdot 10^2$
90	$2.5 \cdot 10^1$	50	n.d.
100	$1.5 \cdot 10^1$	60	n.d.

n.d. – not detected

Table 2. Quantitative changes of *Listeria monocytogenes* in sewage samples on particular days of research.

Days of experiment	Mean number of *Listeria monocytogenes* [CFU·ml^{-1}] at 4ºC	Days of experiment	Mean number of *Listeria monocytogenes* [CFU·ml^{-1}] at 20ºC
0	$9.5 \cdot 10^8$	0	$2.5 \cdot 10^8$
10	$1.5 \cdot 10^8$	10	$9.5 \cdot 10^7$
20	$4.8 \cdot 10^6$	20	$1.0 \cdot 10^5$
30	$1.5 \cdot 10^6$	30	$4.8 \cdot 10^3$
40	$9.5 \cdot 10^5$	40	$2.0 \cdot 10^3$
50	$4.8 \cdot 10^5$	50	$2.5 \cdot 10^1$
60	$1.5 \cdot 10^4$	60	n.d.
70	$9.5 \cdot 10^3$	70	n.d.
80	$2.5 \cdot 10^3$	80	n.d.
90	$1.5 \cdot 10^3$	90	n.d.
100	$4.8 \cdot 10^2$	100	n.d.

n.d. – not detected

At 20ºC, a faster reduction of *Listeria monocytogenes* was observed in the tested water as compared with 4ºC. Initially, the number of cells amounted to $9.5 \cdot 10^8$ CFU·ml^{-1} and fell to the level of $4.5 \cdot 10^7$ CFU·ml^{-1} on the 10th day of the experiment. A gradual decrease in the number of the bacteria was observed in the successive determinations, and on day 30 of

the study their count fell considerably to a value of $1.5 \cdot 10^2$ CFU·ml^{-1}, whereas on the same day of the experiment at 4ºC, microorganisms were isolated in a number of $1.0 \cdot 10^6$ CFU·ml^{-1}. In the penultimate determination, i.e. on day 50, the tested microorganisms were no more isolated (Table 1, Fig. 2).

The effect of temperature on the survival time and elimination rate of the bacteria *Listeria monocytogenes* in meat industry plant sewage was also determined in the experiment. At 4ºC the number of bacteria in sewage in the initial test was $9.5 \cdot 10^8$ CFU·ml^{-1} and it decreased to a value of $4.8 \cdot 10^6$ CFU·ml^{-1} on 20th day of the experiment, and then a gradual reduction in the given microorganisms by one logarithmic unit was observed during the successive days of the study. On the last day of the experiment (day 100) bacterial cells were isolated at a level of $4.8 \cdot 10^2$ CFU·ml^{-1} (Table 2, Fig. 3). In contrast, in sewage with a temperature of 20ºC a considerably faster elimination rate of *Listeria monocytogenes*, since as early as on day 50 of the study a considerable decrease in the number of the bacteria ($2.5 \cdot 10^1$ CFU·ml^{-1}) was observed in relation to the initial sampling ($2.5 \cdot 10^8$ CFU·ml^{-1}). On day 60 of the experiment the tested microorganisms were no more isolated, whereas on the same day of the experiment at 4ºC a concentration of listeria at a level of $1.5 \cdot 10^4$ CFU·ml^{-1} was observed (Table 2, Fig. 4).

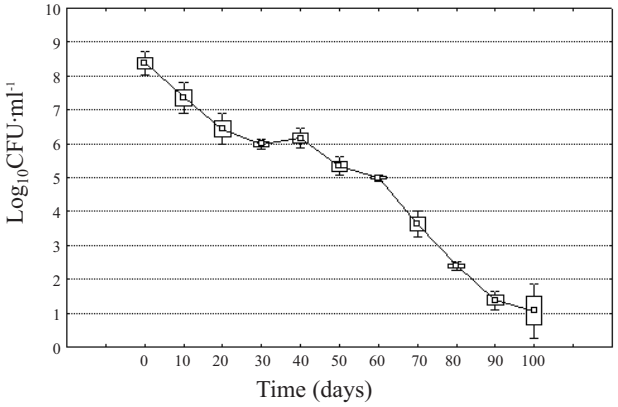

Fig. 1. Survival of *Listeria monocytogenes* in water at 4ºC. The error bars indicate standard deviation of triplicate experiments.

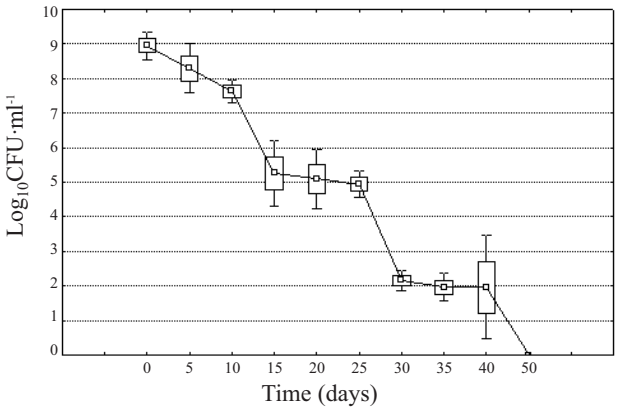

Fig. 2. Survival of *Listeria monocytogenes* in water at 20ºC. The error bars indicate standard deviation of triplicate experiments.

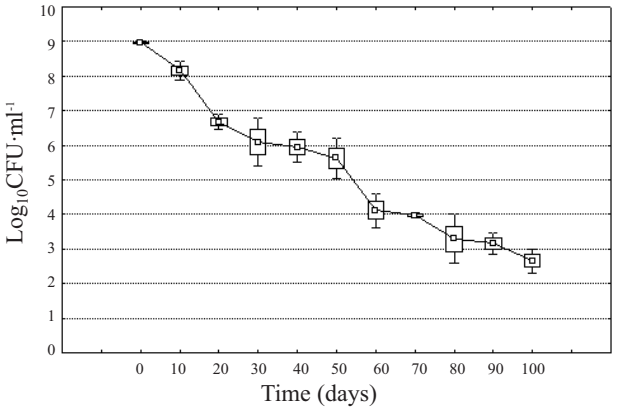

Fig. 3. Survival of *Listeria monocytogenes* in sewage at 4ºC. The error bars indicate standard deviation of triplicate experiments.

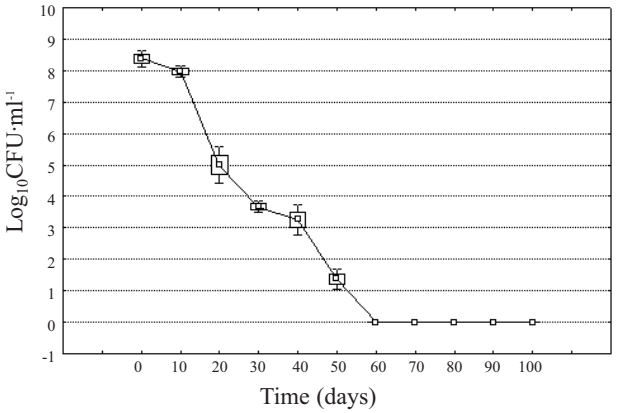

Fig. 4. Survival of *Listeria monocytogenes* in sewage at 20ºC. The error bars indicate standard deviation of triplicate experiments.

The obtained results of the present study indicate that during this experiment the bacteria *Listeria monocytogenes* survived considerably longer in water environment at 4ºC as compared with 20ºC. Similar tendencies were noted by Hansen et al. [1], who found a definitely slower dying rate of listeria cells in natural fresh and sea water at low temperatures of incubation. The relation showed by these authors corresponds to the results of our own study, where a daily rate of bacteria population reduction, according to the analysis of regression, in water at 4ºC was 0.07 log, whereas at 20ºC it was higher and took a value of 0.19 log (Fig. 5). On that basis, it may be assumed that a temperature of 4ºC has a stabilizing effect on bacilli of *Listeria monocytogenes*. Undoubtedly, an explanation of this phenomenon should be sought in the psychrotrophic nature of the determined bacteria [29]. Many authors show their particular predisposition to growth in low temperatures [30-32]. According to reports of Bolton and Frank [33], this is related to adaptive abilities of listeria bacilli, which is confirmed also by the study by Membre et al. [34]. From a

review of the literature data it follows that the average minimum temperature of growth for these bacteria ranges from 1 to 3ºC [12], whereas listeria bacilli can survive even at temperatures below 0ºC [35]. According to Ryser and Marth [12], adaptation of listeria bacilli to the low temperature conditions causes their slowing down of all the vital processes. As a consequence, listeria show more effective resistance, among others to the lack of nutrients, that can allow them to survive longer in the water environment. This reasoning is reflected in the results of the author's own study. The theoretical maximum survival time of listeria bacilli at 4ºC, determined on the basis of the regression line equation, amounted to 120 days, whereas at 20ºC it was considerably shorter and amounted to 47 days. The experiment carried out by Hansen et al. [1] indicates that the bacteria *Listeria monocytogenes* in the natural fresh water at 5ºC were detected to the maximum day 40 of the study, whereas at 20ºC their presence was no longer observed after day 19.

In the author's study concerning sewage from the meat industry, the elimination rate of the bacteria *Listeria monocytogenes* at 4ºC determined on the basis of the regression line was 0.06 log a day (Fig. 6), whereas the maximum survival time calculated amounted to 141 days. At 20ºC, in turn, the daily rate of dying of bacteria cells was higher by 0.08 log as compared with 4ºC and it took a value of 0.14 log (Fig. 6), and the maximum survival time of microorganisms in the tested sample was 61 days. From the above results it follows that the storage temperature of sewage samples determined the dynamics of inactivation of the tested microorganisms, just as in the analyzed water samples. Budzińska and Wroński [28] in the study over the effect of pH on the degree of survival of *Listeria monocytogenes* in sewage from meat industry plants, found that those bacteria showed the longest survival time in raw sewage, where the theoretical time of their survival according to the statistical calculations amounted to 38 days. The reaction of raw sewage during the experiment stayed within the range 6.39-8.32 and it may be concluded that it created the best living conditions for listeria. In alkalized (pH value of 8.20-8.61) and acidified (pH value of 5.18-7.25) sewage, the tested bacteria were able to survive 33 and 25 days, respectively.

A number of biotic and abiotic factors exist in the water environment and sewage that have a direct effect on the survival time of *Listeria monocytogenes*. Besides temperature and pH, a reduction in listeria cells can also depend on exposure to UV and the content of nutrients. Additionally, a faster elimination of *Listeria monocytogenes* is also affected by the phenomenon of predation, mostly at a higher temperature of incubation, since organisms feeding on bacteria, i.e. protozoa, can better develop in the environment at that time [1, 7].

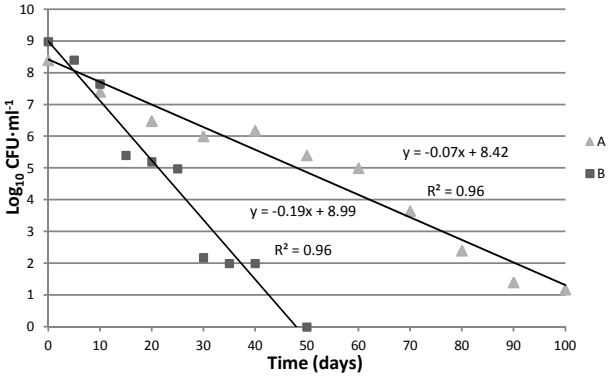

Fig. 5. Regression line equations characterizing the inactivation rate of *Listeria monocytogenes* in water at 4ºC (A) and 20ºC (B).

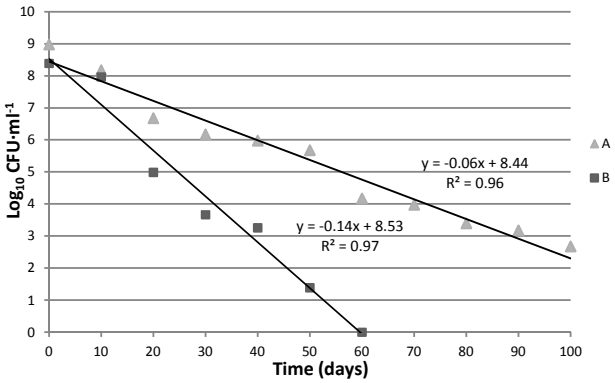

Fig. 6. Regression line equations characterizing the inactivation rate of *Listeria monocytogenes* in sewage at 4ºC (A) and 20ºC (B).

Conclusions

1. On the basis of the research conducted it was found that the bacteria *Listeria monocytogenes* underwent a gradual elimination in water and sewage from meat industry plants, and the inactivation rate of these microorganisms was distinctly dependent on the temperature conditions of storage of the tested samples.
2. It was proved that a temperature of 4ºC affected a longer survival time of listeria cells, where the maximal time of survival of microorganisms in water and sewage calculated on the basis of regression analysis amounted to 120 and 141 days, respectively.
3. Considering the survival rate of *Listeria monocytogenes* in the sanitary and epidemiological aspect, it should be stressed that according to the literature reports, a longer survival time of these bacteria observed in this experiment at 4ºC is particularly significant, owing to the highest virulence of listeria cells at low incubation temperatures.
4. The results obtained in the experiments point out the necessity of constant monitoring of sewage from meat industry plants, as well as surface waters, within the range of effect of those facilities, for pathogenic and potentially pathogenic microorganisms.

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