Polish J. of Environ. Stud. Vol. 17, No. 5 (2008), 827-833

Letter to Editor

Effect of pH on Survival Rate of *Listeria monocytogenes* in Sewage from Meat Processing Plant

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Received: 14 February, 2008
Accepted: 19 May, 2008

Abstract

The aim of the present paper was to evaluate the survival rate of *Listeria monocytogenes* in meat processing industry sewage, depending on the pH under laboratory conditions. In the present experiment we determined the time of survival of listeria bacilli in three kinds of sewage: crude, alkalized and acidified. The present research shows that *Listeria monocytogenes* survived longest in crude sewage where, as calculated with the use of regression analysis, theoretical time of survival was defined as 38 days, while in the alkalized sewage and acidified sewage the time was 33 and 25 days, respectively. Besides, the present experiment also investigated the potential of listeria bacilli survival in sewage under strongly acidic and alkaline conditions.

Keywords: *Listeria monocytogenes*, pH, survival rate, sewage from the meat industry

Introduction

Sewage from meat processing plants, due to a large load of pollutants, are a potential hazard source for aquatic environments. The contamination of abattoir effluents with numerous pathogenic microorganisms, among which *Listeria monocytogenes* can be found, constitutes a serious sanitary problem [1, 2]. According to the literature, the frequency of isolation of this bacteria from sewage is high and may range from 60 to 92.5% [3, 4]. The considerable percentage of listeria in sewage results in their widespread presence in water environments. The presence of these microorganisms was confirmed in rivers, lakes, and mountain streams, as well as in sea-water and ground waters [5]. Hansen et al. [6] indicated the occurrence of *Listeria monocytogenes* in water at a level of 25% at the abattoir impact area. The reports by other authors indicate that the level of water contamination with these bacteria can be considerably varied and range from 3.9 to 62% [7-9].

The long survival time of *Listeria monocytogenes* in water environments is of great importance from an epidemiological point of view. This is related to an ability of this pathogen to multiply and grow under unfavourable conditions for other microorganisms, especially at low temperature and within the wide range of pH [10-13]. Owing to this *Listeria monocytogenes* is able to survive in water even for 300 days, which can, consequently, cause a sporadic or epidemic incidence of listeriosis among people [9, 14]. Mainly elderly people, pregnant women, neonates, people with lowered immunity, suffering from AIDS and neoplasms and patients after organ transplantations are susceptible to disease [15, 16]. The infection usually leads to meningitis, encephalitis, gastroenteritis, septicemia, as well as abortions, stillbirths, or neonates infections [17-19].

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Mortality rates in the course of listeriosis is high and amounts to approximately 30% [15].

The aim of this study was to estimate the survival time of *Listeria monocytogenes* in sewage from the meat processing industry under the influence of different conditions of pH in order to evaluate the usefulness of this method in the process of elimination of the bacteria researched.

Material and Methods

Bacterial Strain and Inocula Preparation

In the present study the strain *Listeria monocytogenes* ATCC 19114 was used, coming from authentic standard strains from referential collections of American Type Culture Collection (Microorganisms KWIK-STIK, Merck). According to recommended growth requirements, the strain *Listeria monocytogenes* ATCC 19114 was collected with a sterile swab and placed onto the Tryptic Soy Agar (TSA, CASO Agar), which was then incubated at 37ºC for 24 hours. After this time single colonies were collected with a sterile loop and inoculated into the liquid medium Tryptic Soy Broth (TSB, CASO Broth) and then incubated at 37ºC for 24 hours.

Experimental Design and Inoculation

At the beginning of the laboratory experiment 5 samples of crude sewage from meat industry were prepared. To alkalize and to acidify the sewage analyzed, sodium hydroxide was added to 2 samples, and sulfurous acid was introduced to 2 further samples, thus establishing two experiments. Experiment I investigated the survival time of *Listeria monocytogenes* in 3 sewage samples crude (initial pH was 6.39), alkalized (initial pH was 8.2) and acidified (initial pH was 5.18), while in experiment II the effect of extreme values of pH on the dynamics of inactivating *Listeria monocytogenes* was defined in 2 sewage samples - acidified (initial pH was 3.0) and alkalized (initial pH was 10.8). The samples were supplied with pre-made suspension of the bacteria researched of 10 ml per each 1000 ml of sewage. The number of *Listeria monocytogenes* in 1 ml (initial sample) was determined in particular samples one hour after establishing the experiment. On successive days of the experiment each sewage sample was examined in three replications. The count of *Listeria monocytogenes* was determined by defining the most probable number of live bacteria (MPN). Sewage samples throughout the experiment were stored at 20ºC.

Microbiological Analysis

The survival rate of *Listeria monocytogenes* in sewage was evaluated in accordance with the following standards: PN-EN ISO 11290-1 and PN-EN ISO 11290-2 [20, 21], with slight modifications which resulted from the character of the samples tested. Microbiological analyses were made according to the following procedure. The first stage covered initial proliferation in selective liquid medium, namely in half-Fraser (Merck) broth with esculin and lithium chloride and the content of Fraser (Merck) additive in a form of ferric ammonium citrate and decreased concentration of factors selectively inhibiting the growth of accompanying bacteria. In the half-Fraser medium decimal dilutions of sewage from 10^{-1} to 10^{-10} were made, and the samples obtained were incubated at 30ºC for 24 hours. After that period the blackening of the medium showed that probably *Listeria monocytogenes* occurred in the samples tested. At the final stage the positive cultures were inoculated onto two selective solid media that inhibited the growth of common gram-negative bacteria and most gram-positive ones: PALCAM agar base (Merck), with selective additive for Listeria according to Van Netten (Merck), containing polymyxin, ceftazidime and acriflavin and onto Oxford agar (Merck), with selective additive Oxford Listeria (Merck), being a mixture of 4 antibiotics and a dye in a lyophilized form. The incubation of inoculated media took place at 37ºC for 24 hours, and in doubtful cases (namely no growth or poor growth at the first reading) up to 48 hours. On PALCAM agar typical colonies of *Listeria monocytogenes* were growing after 24 hours of incubation in a form of small, minute grey-and-green or olive-and-green colonies, from 1.5 mm to 2 mm in diameter, surrounded by the zone of black-dyed medium, sometimes with black-dyed centre, while after 48 hours of incubation they occurred in the form of green colonies 1.5-2 mm in diameter with a characteristic hollow in the middle, surrounded by the zone of black-dyed medium. On Oxford agar after 24 hours of incubation typical colonies of the bacteria researched were growing in the form of small grey colonies up to 1 mm in diameter, surrounded with the blackening zone, while after 48 hours of incubation they were observed in the form of dark grey colonies, often with green-like shade, 2 mm in diameter with a characteristic hollow in the centre and a clear zone of blackening around them. Whenever suspicious colonies were identified on selective agar media, the species of the microorganisms were identified based on the biochemical system API Listeria, where test eleven relied on the determination of β-hemolysis (bioMerieux) [22].

Statistical Analysis

The results of the survival rate of *Listeria monocytogenes* in sewage were verified and then exposed to statistical analysis based on the use of the following model of kinetic bacteria inactivation in time:

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

where:

N – number of bacteria over specific time in sewage,

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

x – time in days,

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

Table 1. Quantitative changes of *Listeria monocytogenes* in crude sewage on particular days of research (Experiment I).

Days of experiment	pH value	Mean number of *Listeria monocytogenes* [CFU ml^{-1}]	Mean number of *Listeria monocytogenes* [log CFU ml^{-1}]	Standard deviation	Standard error
i.s.	6.39	9.5×10^{9}	9.98	0.02	0.01
1	6.55	2.5×10^{8}	8.40	0.19	0.11
4	6.80	4.5×10^{7}	7.65	0.44	0.25
10	7.02	2.5×10^{5}	5.40	0.09	0.05
13	7.24	1.6×10^{5}	5.20	0.03	0.02
17	7.51	9.5×10^{4}	4.98	0.02	0.01
24	7.93	3.0×10^{4}	4.48	0.07	0.04
28	8.32	1.5×10^{2}	2.18	0.15	0.09

i.s. – initial sample

Table 2. Quantitative changes of *Listeria monocytogenes* in alkalized sewage on particular days of research (Experiment I).

Days of experiment	pH value	Mean number of *Listeria monocytogenes* [CFU ml^{-1}]	Mean number of *Listeria monocytogenes* [log CFU ml^{-1}]	Standard deviation	Standard error
i.s.	8.20	9.5×10^{9}	9.98	0.04	0.02
1	8.12	1.5×10^{7}	7.18	0.13	0.08
4	8.03	2.5×10^{6}	6.40	0.10	0.06
10	8.14	1.5×10^{5}	5.18	0.03	0.02
13	8.24	7.5×10^{4}	4.88	0.08	0.05
17	8.37	2.0×10^{4}	4.30	0.11	0.06
24	8.49	1.5×10^{3}	3.18	0.07	0.04
28	8.61	0.7×10^{1}	0.85	0.06	0.04

i.s. – initial sample

Table 3. Quantitative changes of *Listeria monocytogenes* in acidified sewage on particular days of research (Experiment I).

Days of experiment	pH value	Mean number of *Listeria monocytogenes* [CFU ml^{-1}]	Mean number of *Listeria monocytogenes* [log CFU ml^{-1}]	Standard deviation	Standard error
i.s.	5.18	9.5×10^{9}	9.98	0.06	0.03
1	5.30	1.2×10^{7}	7.08	0.04	0.02
4	5.63	4.5×10^{5}	5.65	0.10	0.06
10	6.09	2.5×10^{3}	3.40	0.09	0.05
13	6.42	2.7×10^{2}	2.43	0.09	0.05
17	6.73	1.2×10^{2}	2.08	0.07	0.04
24	6.91	1.5×10^{1}	1.18	0.12	0.07
28	7.25	n.d.	n.d.		

i.s. – initial sample
n.d. – not detected

Table 4. Regression coefficients defining the dynamics of inactivation of *Listeria monocytogenes* in respective sewage samples (Experiment I).

Sample researched	Coefficient a	Coefficient b	r^2	Correlation coefficient	Maximum time of survival in days
Crude sewage	-0.23±0.03	8.77±0.49	0.90	-0.95**	38
Alkalized sewage	-0.25±0.04	8.24±0.56	0.89	-0.94**	33
Acidified sewage	-0.30±0.05	7.60±0.76	0.86	-0.93**	25

** $p<0.01$

Table 5. Quantitative changes of *Listeria monocytogenes* in alkalized sewage on particular days of research (Experiment II).

Days of experiment	pH value	Mean number of *Listeria monocytogenes* [CFU ml^{-1}]	Mean number of *Listeria monocytogenes* [log CFU ml^{-1}]	Standard deviation	Standard error
i.s.	10.80	4.5×10^9	9.65	0.05	0.03
1	10.33	3.0×10^6	6.48	0.14	0.08
7	9.64	1.5×10^1	1.18	0.15	0.09
14	9.15	n.d.	n.d.		

i.s. – initial sample
n.d. – not detected

Table 6. Quantitative changes of *Listeria monocytogenes* in acidified sewage on particular days of research (Experiment II).

Days of experiment	pH value	Mean number of *Listeria monocytogenes* [CFU ml^{-1}]	Mean number of *Listeria monocytogenes* [log CFU ml^{-1}]	Standard deviation	Standard error
i.s.	3.00	4.5×10^9	9.65	0.05	0.03
1	3.14	1.5×10^7	7.18	0.12	0.07
7	3.44	2.5×10^2	2.40	0.09	0.05
14	3.79	n.d.	n.d.		

i.s. – initial sample
n.d. – not detected

The following coefficients were calculated: correlation, determination and regression, as well as the values of standard deviation and standard error. Based on the pattern of regression lines, the theoretical survival time as well as the rate of elimination of *Listeria monocytogenes* in sewage were defined. The analysis of the results obtained was made with statistical software SAS, version 9.1.

Results

Results of quantitative changes of *Listeria monocytogenes* depending on the pH of respective sewage are given in Tables 1-3 (Experiment I).

In crude sewage the number of *Listeria monocytogenes* in the initial research was 9.5×10^9 CFU ml^{-1}, at initial pH of 6.39 and decreased regularly to the value of 1.5×10^2 CFU ml^{-1} on the 28th day of the experiment, where pH was 8.32 (Table 1). The rate of elimination of bacteria, defined based on the linear regression equation, was 0.23 log per day, at a significantly high coefficient of correlation r=-0.95 ($p<0.01$), while the maximum survival time was calculated as 38 days (Table 4).

In the sewage alkalized to the initial pH of 8.2, initial number of *Listeria monocytogenes* assumed the value of 9.5×10^9 CFU ml^{-1} and decreased on the 28th research day to the level of 0.7×10^1 CFU ml^{-1}, while pH of the environment researched was 8.61 (Table 2). According to statistical calculations, daily rate of reduction in detected bacterial cells was slightly higher as compared with crude sewage and was 0.25 log, at highly significant correlation coefficient r=-0.94 ($p<0.01$), while the theoretical survival time calculated based on linear regression equation was 33 days (Table 4).

In acidified sewage the highest rate of elimination of *Listeria monocytogenes* was observed, as already on the 10th day a clear decrease in the number of bacteria (2.5×10^3 CFU ml^{-1}, pH 6.09) was observed as compared with the initial sampling (9.5×10^9 CFU ml^{-1}, pH 5.18). On the 17th day of the experiment these microorganisms were isolated at the number of 1.2×10^2 CFU ml^{-1} (pH 6.73), whereas in crude sewage the value of 1.5×10^2 CFU ml^{-1} (pH 8.32) was found only on the last day of the experiment (28th day). Besides, bacterial cells were still detected on the 24th day of the present experiment at the level of 1.5×10^1 CFU ml^{-1} (pH 6.91), while on the 28th day of research they were not found (Table 3). The regression analysis showed that the daily rate of dying of listeria population was 0.07 log higher as compared with crude sewage and assumed the value of 0.30 log, at highly significant correlation coefficient $r=-0.93$ ($p<0.01$), while the maximum time of survival of bacteria in a given sample was 25 days (Table 4).

Experiment II investigated the survival rate of *Listeria monocytogenes* in alkalized sewage and in acidified sewage. The results of quantitative changes of bacteria depending on the pH of respective sewage are given in Tables 5 and 6. Having added the prepared suspension of bacteria to acidified sewage (initial pH of 3.0) and alkalized sewage (initial pH of 10.8), concentration of *Listeria monocytogenes* was 4.5×10^9 CFU ml^{-1} (initial sample). During the experiment the initial number of the microorganisms researched showed a very fast decreasing tendency for the microorganisms researched, reaching on the 7th day of research in acidified sewage up to pH 3.44 the value of 2.5×10^2 CFU ml^{-1}, while in alkalized sewage the number of the bacteria on the 7th day of the experiment was even lower and reached 1.5×10^1 CFU ml^{-1} at pH of 9.64. On the 14th day of research, both in alkalized and in acidified sewage no bacterial cells were found any more.

Discussion

Listeria monocytogenes is commonly isolated from meat processing plants, where it can persist for months or even years. From the literature it can be concluded that the environment and appliances in the meat processing plant are contaminated to a different extent with these bacteria, and the percentage of positive samples can range from 9 to 67.1% [23, 24]. In sewage from meat processing plants listeria cells are found, which can penetrate into the environment mainly with gastrointestinal contents, animal faeces and washings from production surfaces [1, 25, 26]. Additionally, special attention should be paid on the ineffectiveness of mechanical and biological treatment of sewage from meat processing industry in the elimination of *Listeria monocytogenes*, which might, consequently, contribute to an increasing problem of microbiological pollution of surface waters [5, 27]. From the sanitary point of view, this situation poses a potential epidemiological hazard, all the more so because these bacteria, owing to their particular resistance to unfavourable conditions, are able to survive for long periods of time in water environments, not losing their virulence [7, 9, 11].

In the present experiment the effect of pH on the elimination rate of *Listeria monocytogenes* in sewage from the meat processing industry was defined. The first experiment was made for crude sewage, alkalized or acidified. Based on the regression analysis, the lowest rate of dying was identified for listeria in crude sewage, the number of which was decreasing during the day by 0.23 log. The rate was slightly higher in alkalized sewage, where the daily rate of reduction was 0.25 log, while the fastest process of elimination was found for listeria in acidified sewage, for which the daily dynamics of inactivation was 0.30 log. The present research demonstrated that *Listeria monocytogenes* showed the highest survival rate in crude sewage, where the theoretical survival time calculated based on the linear regression equation was defined as 38 days (Table 4). The reaction of crude sewage throughout the experiment ranged from 6.39 to 8.32, and thus it can be concluded that it created the best habitat conditions for listeria. The results obtained are in agreement with the reports by Galdiero et al. [28], who state that the optimum pH for the growth of the bacteria is neutral of slightly basic. The survival rate of *Listeria monocytogenes* observed in the present experiment at the initial pH of 6.39 corresponds to the data reported by other authors, who stress that only pH below 5.6 shows a destructive effect on bacterial cells, inhibiting the growth and proliferation of this pathogen [19, 29], while the survival time of *Listeria monocytogenes* was slightly shorter in alkalized sewage, where the theoretical survival time was calculated as 33 days (Table 4). The researched sewage clearly showed alkaline pH ranging from 8.20 to 8.61, which must have deteriorated the habitat conditions of these microorganisms. The presence of bacteria population (pH 8.61) identified still on the last day of research coincides with the reports by Capita et al. [17], who claim that inhibiting the development of listeria can be observed when pH approaches the value of 9.6, while among the samples analyzed the shortest survival time of these bacteria was recorded in the acidified sewage with pH ranging from 5.18 to 7.25. As calculated from the linear regression equation, the maximum theoretical time indispensable to inactivate all the listeria cells was defined as 25 days (Table 4). Therefore, any clearly higher rate of elimination of listeria must have been connected with a considerably low pH, but the present results show quite clearly that these bacteria tolerate acidic conditions, which is also confirmed by other authors [10, 30]. As seen from observations reported by George et al. [29], *Listeria monocytogenes* can survive in the environment with pH ranging from 5.23 to 5.45, whereas Cole et al. [31] demonstrated the capacity of listeria to growth in pH of 4.36.

On successive days of the experiment, the growth of pH observed in the samples tested could result from the fact that meat processing industry sewage, due to its specific chemical composition, makes a valuable source of nutrients for numerous microorganisms, which use it for their metabolic processes and thus affect the environment pH modification.

In the second experiment the effect of extreme values of pH on the elimination rate of *Listeria monocytogenes* from

alkalized or acidified sewage was defined. Results of the present research clearly show that listeria can survive in a wide range of pH, which is also reported by Préstamo et al. [12]. In the present experiment in alkalized sewage with the initial pH of 10.8, listeria were isolated still on the 7th day of the experiment (Table 5). The present results correspond with the reports by Czeszejko et al. [5] who claimed that pH even by 11 does not eliminate these bacteria completely, and only contributes to their reduction. A similar opinion is reported also by Vasseur et al. [13], claiming that listeria can show a relatively high resistance to pH, ranging from 10 to 11, while researching the survival rate of *Listeria monocytogenes* in acidified sewage up to the initial pH of 3.0 a lower rate of inactivation of these microorganisms was found as compared with alkalized sewage. This phenomenon can be connected, as reported by Ryser and Marth [9], with the adaptation potential of listeria to acidic conditions, which, to some extent, increases their survival potential. However, according to Parish and Higgins [32], the possibility of survival of *Listeria monocytogenes* at a very low pH ranging from 3.0 to 4.5 is very limited and leads to a fast reduction of bacterial cells. Failure in isolating *Listeria monocytogenes* in alkalized and acidified sewage on day 14 of the experiment might have been related to forming viable but non-culturable (VBNC) cells by these microorganisms. Bacteria turning to VBNC state retain their virulence and undergo changes that allow them to survive under environmental stress conditions [33].

It should be noted that besides the reaction, the survival time of *Listeria monocytogenes* in sewage is also affected by other parameters, most important of which are temperature, the content of easily available nutrients, insolation and salinity level [34-36]. Also, biotic factors such as the activity of indigenous microflora and the presence of protozoa or bacteriophages may play an important role in the process of elimination of listeria cells [6, 35].

In summary, it follows from research that *Listeria monocytogenes* is able to survive in meat industry sewage within a wide range of pH. The method applied in the experiment, based on using different pH values, is not an effective means of eliminating listeria from the tested sewage which, discharged into surface waters without being properly treated, might have a negative effect on their sanitary state and, consequently, pose a potential health hazard for people and animals. Thus, there is a need for constant monitoring of *Listeria monocytogenes* presence in sewage from meat processing plants and carrying out its final disinfection in order to eliminate the risk of the pathogen's spread in the environment.

Conclusions

1. In crude sewage with the pH that was most similar to the optimum one for listeria cells, their survival time was the longest and amounted to 38 days.
2. No effect of pH variability on the survival time of *Listeria monocytogenes* in sewage was found within the tested range of this parameter. The survival time of listeria under different pH conditions which was determined experimentally was at a similar level in relation to the theoretical survival time of the bacteria in crude sewage.
3. Inactivation period of *Listeria monocytogenes* cells in the sewage researched poses a health hazard for people and animals, thus its disinfection should be considered.

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