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Anthropogenic impact on the presence of *L. monocytogenes* in soil, fruits, and vegetables

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Abstract The aim of this study was to determine the prevalence of *Listeria* sp. and *Listeria monocytogenes* in soil samples with reference to type of fertilizers (natural and artificial) and distance from places intensively exploited by men, as well as to determine the relationship between the presence of *L. monocytogenes* in the soil and in fruits and vegetables. The examined 1,000 soil samples originated from 15 different areas, whilst 140 samples of fruits and 210 samples of vegetables were collected from those areas. *L. monocytogenes* was isolated only from 5.5 % of all soil samples coming exclusively from meadows intensively grazed by cattle (27.8 %) and areas near food processing plants (25 %) and wild animal forests (24 %). *Listeria* sp. and *L. monocytogenes* were not present on artificially fertilized areas and wastelands. *L. monocytogenes* was detected in 10 % of samples of strawberry, 15 % of potato samples, and 5 % of parsley samples. Our data indicate that *Listeria* spp. and particularly *L. monocytogenes* were found in the soil from (1) arable lands fertilized with manure, (2) pasture (the land fertilized with feces of domestic animals), and (3) forests (again, the land fertilized with feces of animals, not domestic but wild). The bacteria were not detected in the soil samples collected at (1) artificially fertilized arable lands and (2) wastelands (the lands that were not fertilized with manure or animal feces). Moreover, a correlation was determined in the presence of *L. monocytogenes* between soil samples and samples of the examined fruits and vegetables.

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Introduction

The genus *Listeria* comprises nine species: *Listeria monocytogenes*, *Listeria ivanovii*, *Listeria innocua*, *Listeria welshimeri*, *Listeria seeligeri*, *Listeria grayi*, *Listeria marthii* (Graves et al. 2010), *Listeria rocourtiae* (Leclercq et al. 2010) and *Listeria weihenstephanensis* (Halter et al. 2012), among which only *L. monocytogenes* is pathogenic to humans and *L. ivanovii* to animals causing listeriosis. *L. monocytogenes* is especially dangerous to humans from the group of young, old, pregnant, and immunocompromised patients. About 17 % of listeriosis cases occur during pregnancy (www.fda.gov/downloads/Food/FoodborneIllnessContaminants/UCM312787.pdf). *L. monocytogenes* causes encephalitis and aseptic meningitis. In the USA, an estimated number of 1,600 persons become seriously ill with listeriosis each year. Although *L. monocytogenes* is not the most numerous bacteria found in food, it is considered to be much more dangerous in comparison with other pathogens, since 20–30 % of all listeriosis cases are fatal (Reissbrodt 2004).

The first information on the presence of *L. monocytogenes* in soil was published in 1960 by Welshimer. The object of Weis and Seeliger (1975) research were plants (corn, wheat, and oat) as well as soil from the area of their tillage. Almost 10 % of corn and 13 % of other plants were contaminated by *L. monocytogenes*. Strains of these bacteria were isolated three times more often from nonarable fields (44 %) than from the ones of intensive cultivation (12.5 %). *L. monocytogenes* was suggested to occur in the relation soil–plants–animals–food. Since then, it is generally believed that *L. monocytogenes* is permanently present in soil (Weis and Seeliger 1975). Some scientists claim that *L. monocytogenes* is as common in nature as lactic fermentation bacteria. *Listeria* strains have been isolated from more than 50 hematocryal and homeothermic species including arthropods, fish, and birds (Hird and Genigeorgis 1990; Hellstrom et al. 2007; Szymczak et al. 2013; Zaremba and

Borowski 2001). The presence of *L. monocytogenes* in wandering rodent populations is of great epidemiological importance because of its contact with breeding animals, humans, and consumed food. This bacterium was isolated from dairy products, meat, fruits, and vegetables (Hayes et al. 1986; Donelly 1990; Wang and Slavik 2005). Recent works by Nightingale et al. (2004) indicate that *L. monocytogenes* was most often isolated from farms of intensive cattle graze. In addition, a research by Sauders et al. (2012) shows that *Listeria* sp. and *L. monocytogenes* bacteria were occurring only in samples originating from urban areas.

There are literature reports on several cases of listeriosis after eating raw vegetables. The most famous is the case of listeriosis, whose source of infection was cabbage fertilized with manure from sheep suffering from listeriosis—34 cases of perinatal listeriosis and 7 cases in adults (Schlech et al. 1983). There have also been cases of listeriosis in the USA after eating salads prepared of raw vegetables (Ho 1986) and in Sweden after eating mushrooms (Nguyen-the and Karlin 1994). Recent outbreaks of listeriosis were reported in the USA after consumption of melons, which resulted in 30 fatal cases (MMWR 2011). The source of *L. monocytogenes* contamination of fruits and vegetables is the soil. The main reasons of contamination include the use of contaminated manure to fertilize the soil and the purity of water used for washing vegetables and fruits. The quality of these raw materials is evaluated by the consumer based on the appearance and freshness. Despite reports about *L. monocytogenes* occurrence in food, there is little information about its presence in soil and about characteristics of the sampling sites. Therefore, the aim of this study was to determine the prevalence of *Listeria* sp. and *L. monocytogenes* in soil samples with reference to:

- Land management,
- Type of fertilizers: natural (manure produced from domestic animals and wildlife) and artificial (minerals produced in chemical processes),
- Distance from places intensively exploited by men, and
- Determine a relationship between the presence of *L. monocytogenes* in the soil and in fruits and vegetables. Achieving this aim will allow to determine a correlation in the presence of *L. monocytogenes* between soil and fruit/vegetable samples.

Materials and methods

Soil

In the years 2008–2011, 1,000 soil samples were examined, all collected in Poland (Table 1). The samples were collected in the same climate, the same latitude, and time of year, with an area of about 250 km^2. That particular territory covers small areas with intensive cattle pasture, but mostly includes artificially fertilized agricultural areas and waste grounds. That information was gathered through a survey carried out with farmers. The samples were taken from areas intensively exploited by men (arable lands, areas near food processing plants, meadows intensively grazed by cattle), forests, confined wild animal breeding (boars, roes, deer, and fallow deer), as well as from wastelands. In the case of the area near food processing plants, samples were collected at a distance of 150 m from the plant, whereas in the case of meadows A and B—at a distance of 500 m. The distance between meadows A and B was 80 km. They differed in the species of cattle that was grazing on (A—dairy cattle, B—meat cattle). The samples were collected from the surface with a sterile spatula into sterile plastic bags, delivered to a laboratory within 1–2 h and analyzed.

Fruits and vegetables

We studied 140 samples of fruits, including: blueberries (20 samples), blackberries (20), raspberries (40), and strawberries (60), and 210 samples of vegetables, including: beetroot, cabbage, carrot, parsley, tomato, lettuce, and potato (30 samples of each). Samples of fruits and vegetables were collected from the same areas the soil samples were collected from naturally and artificially fertilized garden plots and orchards. Before the analysis, the fruits were thoroughly washed under running water, then stalked (for strawberries), while the vegetables were peeled with a sterile knife for vegetables (beetroot, carrot, parsley, and potato) and then washed under running water.

Reference strains of *L. monocytogenes*

The control samples were 10 reference strains of *L. monocytogenes* constituting a positive control for multiplex PCR. The reference strains of *L. monocytogenes* (1/2 A, 1/2 C, 125 wł, 150 wł, and OSP 3) came from the collection of the Dipartimento di Scienze degli Alimenti in Italy. The strains CEB 3176, CEB 3609, and CEB 3628 were from the Institut Pasteur in France and BB 45262, BB 4581 from Lund University in Sweden.

Listeria isolation from soil samples

Each 25-g weighed portion of soil sample was transferred to a flask with 225 mL Half Fraser without selective agent (Fraser Broth 835, Oxford, England). The flask was shaken with arid glass pearls in an orbital shaker for 10 min. The samples were incubated for 24 h at 30 °C. Then 1 mL of the culture was transferred to 10 mL Fraser with Fraser Selective Supplement (SR 156, Oxoid). Simultaneously, a culture into

Table 1 Incidence of *L. monocytogenes* in soil, fruit and vegetables

Type of soil	No. (%)[a]	Fruit	No. (%)[a]	Vegetables	No. (%)[a]
Arable lands:					
Natural fertilizing	173 (1.2)	strawberry	20 (10.0)	beetroot	10 (0)
				cabbage	10 (0)
				carrot	10 (0)
				lettuce	10 (0)
				parsley	10 (5.0)
				potato	10 (15.0)
				tomato	10 (0)
Artificially fertilized	173 (0)	strawberry	20 (0)	beetroot	10 (0)
		raspberry	20 (0)	cabbage	10 (0)
				carrot	10 (0)
				lettuce	10 (0)
				parsley	10 (0)
				potato	10 (0)
				tomato	10 (0)
Wastelands	120 (0)				
Garden plots	47 (10.6)	strawberry	20 (10.0)	beetroot	10 (0)
		raspberry	20 (0)	cabbage	10 (0)
				carrot	10 (0)
				lettuce	10 (0)
				parsley	10 (5.0)
				potato	10 (15.0)
				tomato	10 (0)
Orchards	18 (0)				
Meadows					
Intensive dairy cattle graze (A)	36 (27.8)				
Intensive meat cattle graze (B)	44 (13.6)				
Small grazing cattle (some units)	20 (0)				
Wastelands	78 (0)				
Confined wild animal breeding (boards, deer and fallow deer)	32 (15.6)				
Areas near food processing plants	36 (25.0)				
Forests					
Deciduous	72(12.5)	blackberry	20 (0)		
Coniferous	72 (2.8)	blueberry	20 (0)		
Around the lake	50 (0)				
Area hunting districts	29 (24.0)				

[a] Number of analyzed samples/ percent of positive test is given as the proportion of PCR method

single colonies was performed on the Listeria Selective Agar (LSA) medium (CM 856, Oxoid, England) with *Listeria* Selective Supplement (SR 140, Oxoid). First, the samples were incubated on the Fraser medium and then on the LSA medium for another 48 h at 37 °C (Scotter et al. 2001).

Listeria sp. and *L. monocytogenes* strains identification

Five colonies typical of *Listeria* on the LSA medium were randomly chosen from each sample for further identification. The sample where at least one colony was identified with biochemical tests as *Listeria* sp. and as *L. monocytogenes* in the PCR reaction was acknowledges as positive. Strains were identified to the *Listeria* genus (stained preparation with Gram method, catalase, oxidase, motility at 20 °C and 37 °C) and to *L. monocytogenes* species by API tests (BioMerièux, France).

Microbial DNA isolation

DNA isolation was performed from a 24-h microbial culture in BHI broth using Genomic Mini AX Bacteria kits (A&A Biotechnology, Poland). The concentration and purity of

DNA were checked spectroscopically (NanoDrop DN-1000, Biotech, USA).

Multiplex PCR

Identification to the genus *Listeria* was performed using primers specific for the 16S rDNA sequence fragment: U1 and LI1 (Border et al. 1990). The primers iap1 (5′ -CgA ATC TAA Cgg Ctg gCA CA- 3′) and iap2 (5′ -gCC CAA ATA gTg TCA CCg CT- 3′) specific for the *iap* gene fragment (Jaton et al. 1992) were used for species identification to *L. monocytogenes*. The sizes of amplified DNA sequences were 938 bp for 16S rDNA fragment and 287 bp for *iap* fragment. Due to a high number of strains being identified, a multiplex PCR was performed. Multiplex PCR reaction was carried out in a volume of 50 μL using Mastercycler Gradient (Eppendorf). Each reaction mixture contained 500 mmol/L KCl, 100 mmol/L Tris–HCl (pH 8.3 at 25 °C), 2.5 mmol/L $MgCl_2$, 0.3 mmol/L of each nucleoid, 30 pmol/L of each primer, 2.5 U Taq DNA polymerase (Eppendorf), and 5 μL DNA template. The thermal profile consisted of the following stages: preliminary denaturation—60 s at 95 °C and 35 cycles including: denaturation—30 s at 94 °C, primers annealing—20-s at 51 °C and extension 30 s at 72 °C. The amplification ended with extension 8 min at 72 °C.

Agarose gel electrophoresis

Eight microliters of each reaction product with 0.037 g of Bromophenol Blue (ICN Biomedicals INC, USA) in a 1.5 % sucrose solution were separated electrophoretically under standard conditions (5 V/cm) in a 2 % agarose gel (Prona Agarose Plus). The gel was stained with ethidium bromide (0.5 μg/mL) in TBE buffer, observed in UV light and archived (GelDoc, BioRad). The size of the product was compared with the mass marker XVI (Roche, Germany).

Statistical analysis

The results obtained from soil, fruits, and vegetables were analyzed statistically using Spearman's correlation with a 0.05 significance level in StatSoft Statistica v 9.0 (Statsoft, Tulsa, OK, USA).

Results

L. monocytogenes in soil

The presence of *L. monocytogenes* was determined in 5.5 % of 1,000 examined soil samples acknowledged by PCR multiplex reaction, as shown in Table 1. *L. monocytogenes* was isolated only from the areas intensively exploited by men including areas near food processing plants (25 %), meadows intensively grazed by cattle (13.6 – 27.8 %), and garden plots intensively fertilized with manure (10.6 %).

One hundred seventy-three organically fertilized soil samples, 173 artificially fertilized soil samples, and 120 wasteland samples were examined. *L. monocytogenes* was isolated only from the areas fertilized organically (1 %), whereas both *Listeria* sp. and *L. monocytogenes* did not occur in artificially fertilized areas and wastelands (Table 1).

Studies have shown that out of the 36 soil samples collected in the immediate vicinity of a meat factory, *L. monocytogenes* was isolated in 25 % of the samples that originated exclusively from direct proximity to the main entrance of the plant. *Listeria* sp., and *L. monocytogenes* were not isolated from the samples located more than 50 m away from the food processing plant (data not shown). Out of the 36 samples collected on the meadow land A and B, the presence of *L. monocytogenes* was detected in 28 and 14 % of the samples, respectively. The presence of *Listeria* species and *L. monocytogenes* was confirmed on the areas most frequently used by the livestock (near water holes and feeders). *Listeria* sp. and *L. monocytogenes* were only isolated from the areas where cattle was grazing intensively, whereas *Listeria* was not isolated in the distance of 150 m from the grazing land. Similarly as in the case of meadow A, *L. monocytogenes* was only isolated from the area where the cattle was grazing on (about 200 heads of cattle). In the proximity, there were meadow wastelands and forests, where not even a single *Listeria* colony growing characteristically on LSA was isolated. The type of cattle had no influence on the presence of these bacteria.

L. monocytogenes in fruit and vegetables

Of the 140 tested samples of fruits, *L. monocytogenes* was confirmed only in strawberries (10 %) from naturally fertilized land and allotments (Table 1). Using the multiplex PCR technique, *L. monocytogenes* was confirmed in the samples of parsley and potatoes coming from allotments and agricultural land fertilized naturally, i.e., 5 and 15 %, respectively (Table 1). In other samples, *Listeria* sp. and *L. monocytogenes* were not detected. Correlation analysis showed a significant ($P<0.05$) relationship between the presence of *L. monocytogenes* in garden plots and strawberry (Rho = 0.271). The correlation between the fertilized land and parsley, potato, and strawberry was weak, respectively 0.086, 0.042, and 0.009. In other cases, no correlation occurred, because all data in the variable had a zero value. The high rho value indicates a high risk of contamination of fruits and vegetables grown in the soil with *L. monocytogenes* bacterium.

Discussion

"*Listeria* is present in the environment worldwide". This statement is often used by researchers dealing with *Listeria*. Publications from the 1970s are in fact the only works describing *Listeria* presence in soil (Weis and Seeliger 1975; Welshimer 1968; Welshimer and Donker-Voet 1971). In contrast, in recent years, *L. monocytogenes* has been demonstrated to occur only in strictly specified types of soil and its presence to be linked with the use of manure as an ecological crop or has been isolated from areas of farms with intensive cattle graze (Nightingale et al. 2004). Alike results were achieved in the present study, where *Listeria* sp. and *L. monocytogenes* were isolated from soil intensively fertilized with manure.

Soils samples were collected from areas differing in the type and class of soil; however, previous investigations did not demonstrate those factors to affect the occurrence of *L. monocytogenes* (Kwiatkowska 2008). In addition, the study demonstrated that *Listeria* sp. and *L. monocytogenes* did not occur on uncultivated agricultural and remote areas of larger agglomerations of people. On the contrary, studies by Sauders et al. (2012) showed that members of the genus *Listeria* were not only common in urban and natural environments but also showed species- and subtype-specific associations with different environments and areas. Similar findings were obtained in a research by Zaytseva et al. (2007) that was performed on 76 soil samples from territories of east Russia. *Listeria* sp. and *L. monocytogenes* were not isolated. The author emphasizes that those areas are distant from large urban agglomerations, food processing plants, or animal breeding farms.

The presence of *L. monocytogenes* near food processing plants is not surprising since other authors had earlier reported on a serious problem with *Listeria* on areas of meat plants and others plants connected with food processing. In Tompkin's research (Tompkin 2002), *L. monocytogenes* was isolated from food processing plant's floor. On that account, one can assume that *Listeria* was "carried on shoes" of workers. Earlier, *L. monocytogenes* was isolated from smears from floors, walls and work surfaces of a production hall. Our study confirmed the presence of *L. monocytogenes* in 25 % of soil samples collected from the area near the food processing plant. Our study demonstrated that *L. monocytogenes* was isolated from soil at the distance of 50 m away from the meat processing plant (data not shown). The sources of contamination with *L. monocytogenes* are animal feces left with meat as a result of improperly conducted preliminary treatment. Further on, the bacteria permeate into production halls and transferred onto shoes, etc. On the other hand, the occurrence of *L. monocytogenes* in such a close proximity to the main entrance to the production hall is due to the washing of vehicles transporting carcasses of slaughter animals after unloading Kwiatkowska (2008).

In this work, we examined a specific territory of enclosed wild animal breeding (boars, deer, fallow deer). This was a 20-ha area, and population density was about 100 animals per ha. The presence of *L. monocytogenes* was determined in 15.6 % of the examined samples. Similar findings were obtained by Kalorey et al. (2006), who isolated *L. monocytogenes* from 16 % of feces samples from animals living in a zoo in India. Bauwens et al. (2003) demonstrated *L. monocytogenes* presence in 14 (7 %) feces samples of different wild animals (including 6.7 % mammals and 8.6 % birds) living in a zoo in Antwerp. Research on reindeer feces samples show that wild animals living in low-populated areas, e.g., northern Norway are Listeria-free (Aschfalk et al. 2003). The author emphasizes that those territories are very extensive and reindeers are wandering animals, therefore the possibility of infection of one animal from another is quite low. A potential source of *L. monocytogenes* strains is silage, which is often used to feed animals. Fenlon (1996) and Roberts and Wiedmann (2003) showed a lack of *L. monocytogenes* in feces of animals fed with green fodder, in contrast to animals fed with silage. In turn, Nightingale et al. (2004) examined 504 soil samples from 24 farms of intensive cattle graze and the presence of *L. monocytogenes* was proved in 120 of them (24 %). Their data suggest that (1) the epidemiology and transmission characteristics of *L. monocytogenes* differ between small ruminant and cattle farms; (2) cattle contributed to amplification and dispersal of *L. monocytogenes* into the farm environment; (3) the ruminant, and particularly the bovine, farm ecosystem maintains a high prevalence of *L. monocytogenes*, including subtypes linked to human listeriosis cases and outbreaks, and may thus constitute a significant natural reservoir for *L. monocytogenes*; and (4) *L. monocytogenes* subtypes may differ in their abilities to infect animals and to survive in farm environments.

Similar findings were obtained in our research in the case of samples collected from meadows A and B, 27.8 and 13.6 %, respectively. Wesley et al. (2002) showed that *L. monocytogenes* was more often isolated from cattle feces (33 %) than from sheep feces (8 %). The species of cattle had no effect on the occurrence of *L. monocytogenes*. In our study, 218 soil samples tested originated from forests and the presence of *L. monocytogenes* was detected in 8.3 % of these samples. Polish forests are unique in terms of the high number of visitors (people walking, gathering fruits and mushrooms). In addition, wild animals living in forests often go to ploughland in search for food. The sources of soil contamination with *L. monocytogenes* in Polish forests are feces of wild boars and deers. A research by Koronkiewicz (2006) demonstrated that *L. monocytogenes* was confirmed in 60 % samples of wild boar feces and in 43 % samples of

deer feces. In turn, in the soil samples collected from areas of hunting grounds, *L. monocytogenes* was isolated only from the samples originating from areas, where these bacteria were detected in feces of the game.

L. monocytogenes is a microorganism isolated from different foods, including fruits and vegetables, whether fresh, frozen, or otherwise processed. A study by Beuchat (1996) demonstrated the relationship between the use of natural organic fertilizers and the occurrence of *L. monocytogenes* bacteria in raw vegetables. Our study confirmed that the contamination of fruits and vegetables was related to anthropogenic factors (fertilization, irrigation, storage, transport, etc.). It showed that the fruits and vegetables contaminated with *L. monocytogenes* originated from soils fertilized with manure. Worthy of notice is also that these bacteria were isolated only from those raw materials that had contact with the soil, e.g., strawberries, root vegetables, etc. Furthermore, it was found that the occurrence of pathogenic bacteria, including *Listeria* sp. bacilli, was significantly affected by the method of soil fertilization, because the main source of contamination was manure. Also Heisick et al. (1989) showed that 25.8 % of potato samples and 30.3 % of radish samples were contaminated with *L. monocytogenes*. In contrast, Wong et al. (1990) showed that out of 49 tested samples of vegetables, 12.2 % were contaminated with *L. monocytogenes* bacilli. In turn, in our study, the presence of *L. monocytogenes* in strawberries (10 %), parsley (5 %), and potatoes (15 %) may be an indicator of contamination with these bacteria. Due to the increased intake of fresh fruits and vegetables, the promotion of food from the so-called organic farming in Europe, where manure is the basic acceptable applied fertilizer that can be a source of contamination with *L. monocytogenes* and to the increasing number of listeriosis cases, a need emerges for greater control of food products in terms of *L. monocytogenes* presence.

In this study, correlation analyses were performed to assess the relationship between the occurrence of *L. monocytogenes* in soil and in fruits/vegetables. Soil samples were classified according to the two groups on the basis of proportional occurrence of bacteria (Table 1). Vegetables and fruits collected from the examined soils were classified into one of the two groups, depending on the degree of soil contamination. They were also classified based on the proportional occurrence of *L. monocytogenes*. The first group includes soils not cultivated by man or soils fertilized artificially. These soils do not pose a health threat of *Listeria* food poisoning, since these bacteria were not present on fruits and vegetables. The second group includes soils exploited by man for grazing cattle and those fertilized naturally. *L. monocytogenes* was present in 1.2–27.8 % of the samples. Moreover, depending on the type of vegetables and fruits collected from these soils, *L. monocytogenes* was present in 0–15 % of the samples of fruits and vegetables. The statistical analysis show only one significant correlation between the occurrence of *L. monocytogenes* in fruits, vegetables, and soil.

The consumption of fruits and vegetables from soils from the second group poses the high risk of infection with *L. monocytogenes*. Our study indicates that the most frequent cause of soil contamination with *L. monocytogenes* bacteria is manure, which is linked with the transmission of those bacteria to crops. In most cases, it refers to fruits and vegetables having direct contact with the contaminated soil (strawberries, root vegetables, etc.).

A thesis in which authors claim that soil is a natural environment for *L. monocytogenes*, according to present study, appears to be wrong. A total number of 1,000 soil samples and 140 fruit and 210 vegetable samples were examined, which proves reliability of the research made. *Listeria* spp. and particularly *L. monocytogenes* were found in the soil from (1) arable lands fertilized with manure; (2) pasture (the land fertilized with feces of domestic animals); and (3) forests (again, the land fertilized with feces of animals, not domestic but wild). The bacteria were not found in the soil samples collected at (1) artificially fertilized arable lands and (2) wastelands (the lands that were not fertilized with manure or animal feces). There was a correlation between the presence of bacteria in soil and in fruits/vegetables.

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