***Listeria monocytogenes* – danger for health safety vegetable production**

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**Abstract**

The microbiologically contaminated vegetables represent a risk for consumers, especially vegetables without thermal processing. It is known that human pathogen bacteria, such as *Listeria monocytogenes*, could exist on fresh vegetables. The fresh vegetables could become *Listeria*-contaminated if they come in touch with contaminated soil, manure, irrigation water.

The aim of this work was to investigate the presence of *Listeria* spp*.* and *L. monocytogenes* in different kind of vegetables grown in field and greenhouse condition as well as surface and endophytic colonization plant roots of different vegetables species by *L. monocytogenes* in laboratory conditions.

The detection of *Listeria* spp. and *L. monocytogenes* in vegetable samples was done using ISO and PCR methods. The investigation of colonization vegetable roots and detection Listeria-cells inside plant root tissue was done using Fluorescence *in situ* hybridization (FISH) method in combination with confocal laser scanning microscopy (CLSM).

The results showed that 25.58% vegetable samples were positive for *Listeria* spp. and only one sample (carrot) was positive for *L. monocytogenes* out of 43 samples in total collected from field and greenhouse. The strain *L. monocytogenes* EGD-E surface and endophytic colonized carrot root in highest degree while strain *L. monocytogenes* SV4B was the most represented at leafy vegetable plants, such at lettuce (1.68x106 cells/mm3 absolutely dry root) and spinach (1.39x106 cells/mm3 absolutely dry root) root surface.

The cells of *L. monocytogenes* SV4B were visible as single cells in interior tissue of plant roots (celery and sweet corn roots) as well as in the interior of the plant root cell at sweet corn root. The cells of *L. monocytogenes* EGD-E bind to the surface of the plant root and they were less commonly found out on root hair. In the inner layers of the root, those bacterial cells were inhabited intercellular spaces mainly as single cells very close to the larval vessels of root. Our results suggest that *L. monocytogenes* is very good endophytic colonizer of vegetable plant roots.

**Key words:** *contamination of vegetables, Listeria monocytogenes., irrigation water, FISH, CLSM.*

**Introduction**

The large risk for consumers is microbial contaminated vegetables which are eaten in row, without thermal processing. It is known the presence of human pathogen bacteria on fresh vegetables, such as *Listeria monocytogenes* (Shenoy et al., 2017), *Salmonella typhimurium* (Golberg et al., 2011), *Escherichia coli, Campylobacter* spp. etc. These bacteria are known as causers of diseases: listeriosis, diarrhea syndrome, hemolytic uremic syndrome (HUS), salmonellosis.

The *L. monocytogenes* exists in intestinal tract of humans and worm-blooded animals, soil, water, as well as on fresh vegetables (Beuchat, 2002). The fresh vegetables could become *Listeria*-contaminated if they are in touch with soil and manure. The *L. monocytogenes* is able to cause listeriosis at humans. The common symptoms of listeriosis are: fever, muscle pain, serious gastrointestinal problems, but infection could also catch nerve system (headache, confusion, loss of balance).

There is evidence about listeriosis outbreaks (Gaul et al., 2013; Centers for Disease Control and Prevention, 2015) which are caused by consumption contaminated vegetables and fruits (cantaloupe, celery, apples).

The investigation of presence human pathogen bacteria in different vegetable species originated from supermarkets in USA showed that *L. monocytogenes* was detected in 4.7% of analyzed 127 samples in total (Thunberg et al., 2002). There were analyzed 890 fresh vegetables samples in Norwegian and three samples were positive for presence of *L.* *monocytogenes* (Johannessen et al., 2002).

In the undeveloped and developing countries, the presence of human pathogen bacteria on fresh vegetables is bigger than in other countries. Thus, *L. monocytogenes* was detected in 7 samples of total 66 samples of fresh vegetables which were washed and originated from open market in India (Pingulkar et al., 2001).

According to Swaminathan (2001), the infection dose of *L. monocytogenes* is about 100 cells and more per 1 g fresh produce and that small amount of bacterial cells could be cause of disease.

Some investigations showed that human pathogen bacteria, as well as *L. monocytogenes* could exist on the edges of damaged seed’s coat during germination plant seeds. It means that there is possibility for entrance bacteria into the plant (Gorski et al., 2004). It is considered that bacterial flagellum is very important factor of interaction and has positive effect of attachment *L. monocytogenes* to plant tissue (Gorski et al., 2003).

The aim of this work was investigation presence of *Listeria* spp. and *L. monocytogenes* in different kind of vegetables grown in field and greenhouse condition on different locations in Serbia. Another goal was investigation of surface and endophytic colonization plant roots of different vegetables species by *L. monocytogenes* strains in laboratory ‘’monoxenic’’ conditions (one plant species is inoculated by one bacterial strain). Also, the one of the goals was application of Fluorescence *in situ* hybridization (FISH) method in combination with confocal laser scanning microscopy (CLSM) for detection *L. monocytogenes* inside plant tissue.

**Material and method**

**COLLECTING VEGETABLE SAMPLES FOR MICROBIOLOGICAL ANALYSES**

The samples of fresh vegetables were collected in July and August 2015 from agricultural land and plastic greenhouse in Region of Central Serbia (Figure 1). It was collected totally of 43 samples, 16 samples of tomato (*Solanum* *lycopersicum* L.), 13 samples of sweet papers (*Capsicum annuum*), 2 samples of cabbage (*Brassica* *oleracea*), 1 sample of hot paper (*Capsicum* sp.), 1 sample of cucumber (*Cucumis sativus*), 5 samples of potato (*Solanum* *tuberosum* L.), 4 samples of carrot (*Daucus* *carota*) and one sample of parsley (*Petroselinum* *crispum*). Following the Serbian State Legal Regulations for microbiologically safety products (The Official Journal of Republic of Serbia No. 72/2010., 2010), every sample was divided into five units for microbiological analyses. The microbiological analyses for presence of *L. monocytogenes* in fresh vegetable samples were done by standard method EN ISO 11290-2.

**DETECTION OF *LISTERIA***SPP**. IN VEGETABLE SAMPLES**

For detection of *Listeria* spp., 25 g of vegetable sample was enrichment (initial base solution) in semi-concentrated Fraser Broth (Merck, Germany) (Primary Selective Broth) at 30oC for 24 ± 2h followed by transferring the 0.1 ml of initial base solution to 10 ml of Fraser Broth (secondary selective broth) and incubation at 37oC for 48 ± 2h.

The both suspensions of primary and secondary selective broth was inoculated to Palcam Agar (Merck, Germany) and incubated at 37o C in micro-aerobic condition for 24 – 48h. Presence of typical small gray colonies, 1.5 - 2 mm in diameter, with central depression and black zone around considered as *Listeria* spp. colonies.

In order to confirm presence of *Listeria* spp. it was selected five typical colonies and inoculated to Tryptone Soya Yeast Extract Agar (TSYEA) (Merck, Germany) and incubated at 37 oC for 18 - 24h. Confirmation was done according to morphological characteristics of colonies and single bacterial cells after the Gram staining, catalase test and motility test (in *Listeria* Motility Medium (Merck, Germany) after the incubation at 25oC for 2 – 5 days).

**DETECTION OF *LISTERIA MONOCYTOGENES* USING POLYMERASE CHAIN REACTION (PCR)**

TheDNA extraction was done by modified method (Hopwood et al., 1985). The tube with bacterial culture was centrifuged at 4500 rpm for 10 minutes, washed in 500 μl TEN buffer (50 mM Tris-HCl pH 8; 10 mM EDTA pH 8; 50 mM NaCl) and resuspended in 500 μl PP buffer (0,5 M saccharose; 40 mM NH4-acetate; 10 mM Mg-acetate; pH 7) with addition of lysozyme (in concentration of 4 mg/ml). The bacterial cells were incubated at 37°C for 30 minutes, it was added 250 μl 2% Sodium-dodecyl-sulfate (SDS) and suspension was virtexed for 1 minute. The removal of proteins was done by adding 250 μl neutral phenol-chloroform, vortexing for 30 seconds and centrifuging at 13000 rpm for 2 minutes. The supernatant was collected and it was added 3M Na-acetate, pH 4,8 (1/10 volume) and isopropanol (1 volume) in supernatant. The suspension was gently mixed and incubated at room temperature for 5 minutes. The DNA was precipitated by centrifuging at 13000 rpm for 2 minutes. The pellet was washed by cold ethanol (75%), centrifuged at 13000 rpm for 2 minutes, dried and resuspended in 50 μl RNase solution (10mg/ml). This solution was incubated at 37°C for 30 minutes for removal RNA. The obtained DNA was suitable for PCR.

The primers used in this study are based on sequence data of listeriolysin O gene (hlyA LM1 (5’–CCTAAGACGCCAATCGAA-3’) and LM2 (5’–AAGCGCTTGCAACTGCTC-3’) (Mengaud et al., 1988).

The PCR reaction mixture (50μl total volume) contained: 5μl - 10xPCR buffer (10mM Tris-HCl, pH 9.0, 50mM KCl, 0.1% Triton X-100); 5μl - 25mM MgCl2; 250μM - each dNTP; 2U - Taq DNA polymerase; 50pmol - each primer; 5μl - DNA sample.

The amplification parameters were (Ozbey et al., 2006): 1 cycle – 94°C - 5 minutes (initial denaturation); 45 cycles - 94°C - 30 seconds; 45 cycles - 52°C – 1 minute; 45 cycles - 72°C – 1.5 minutes; 1 cycle - 72°C – 7 minutes (final step). The amplified products (10 μl for each sample) were run in 1.5% agarose gel in 1 x Tris-borate-EDTA buffer (electrophoresis). The ethidium bromide was used for staining gel in concentration of 0.5 μg/ml. The PCR products were visualized by UV light and product sizes were compared with ladder 100 bp DNA (Nippon Genetics, Germany).

**EXPERIMENT OF PLANT COLONIZATION BY *LISTERIA MONOCYTOGENES***

The possibility of surface and endophytic colonization root of different vegetable plants by *L. monocytogenes* strains was investigated in laboratory conditions. It was used ’’monoxenic’’ model growing plants which means that one plant species was inoculated by one bacterial strain.

The colonization experiments were done in laboratories of Research Unit Microbe-Plant Interactions, German Research Center for Environmental Health (GmbH), Helmholtz Zentrum Munchen, Germany.

Two *L. monocytogenes* strains were used: *L. monocytogenes* EGD-E and *L. monocytogenes* SV4B. These bacterial strains are part of bacterial culture collection of Helmholtz Zentrum Munchen, German Research Center for Environmental Health (GmbH), Research Unit Microbe-Plant Interactions. *L. monocytogenes* SV4B was selected such as representative human pathogen bacteria because it is very common in human infections. The other strain (*L. monocytogenes* EGD-E) was selected because it is common strain which is used in laboratory experiments.

The colonization plants by *L. monocytogenes* was investigated at next model species of vegetables: lettuce (*Lactuca sativa*); spinach (*Spinacia oleracea)*;parsley (*Petroselinum crispum*); carrot (*Daucus carota* subsp. *sativus*); celery (*Apium graveolens*); tomato (*Lycopersicon esculentum*); sweet corn (*Zea mays* var. *saccharata*).

The *L.* *monocytogenes* strains were cultivated on selective Oxford Agar (Merck, Germany) with addition specific supplement (Fluka, Switzerland) at 40 ⁰C during the 24h. After incubation, the typical colonies of *L. monocytogenes* (tiny, white and surrounded by a dark zone) were picked up and transferred in the Falcon tubes with 20 ml of Brain Heart Infusion (BHI) Broth (Difco, USA). The tubes were incubated with shaking overnight at 37 ⁰C. The OD was measured for both *L.* *monocytogenes* strains. The final concentration for *L. monocytogenes* EGD-E was adjusted ≈106CFU/1ml PBS and for *L. monocytogenes* SV4B was adjusted ≈ 107 CFU/1ml PBS. Those concentrations of bacterial suspension were used for initial plant inoculation.

**INOCULATION MODEL PLANTS BY *LISTERIA* *MONOCYTOGENES***

The plant seeds for experiment were certified and originated from Germany. The seed surface sterilization was done according to the Burun (2002). The germination of seeds till appearing cotyledons, check seeds sterility before bacterial inoculation and planting were done as Kljujev et al. (2018) described. The model plants were grown in sterile quartz sand in an axenic model system (Kljujev et al., 2018)**.** The inoculated model plants were grown 21 days in sterile conditions at room temperature and normal light mode. After three weeks, the plants removed from sand and root system was cut in sterile conditions. The plant root system was washed in sterile Phosphate-buffered saline (PBS) dilution for removing sand particles and weak attached bacterial cells.

**FLUORESCENCE *IN SITU* HYBRIDIZATION (FISH)**

The Fluorescence *in situ* hybridization (FISH) method was applied for both *L. monocytogenes* strains (EGD-E and SV4B). This method was used for detection sequences of nucleic acid using phylogenetic fluorescently labeled oligonucleotide probes that specifically hybridize the complementary target sequence within the bacterial cell (Moter and Gobel, 2000; Eldor 2007; Blomme and Handler, 2009). This method was also applied for all experimental plants roots which were inoculated by *L. monocytogenes* (EGD-E; SV4B).

The protocol for FISH method included: root sample fixation; sample preparation and pretreatment step; hybridization; (binding specific probes for determination the specific target sequence); washing samples (to eliminate unbundled probes); confocal microscopy and interpretation results.

Before hybridization, the root samples had to be fixed because it helps that sample becomes permeable and fluorescence probes are able to get into bacterial cells. The fixation plant root for investigation *Listeria* strains was done by Ethanol fixation method. It was used absolute ethanol. First, it was harvested plant root and removed adhering soil particles. After that, the root was washed with 1 x PBS. It was prepared a 1:1 (vol./vol.) fixation solution EtOHabsolute/1 x PBS in a 50 ml Falcon Tube. The root was gently added to the fixation solution. Then, it was done incubation for 2h at 4°C. At the end, it was discarded fixation solution and root was washed 3 times with 1 x PBS. The root was stored in 1:1 mixture of 1 x PBS/EtOHabsolute at -20°C (Amann et al., 1990).

In this step of FISH procedure, the hybridization buffer was added to the sample containing fluorescence probes which are complementary to the target site on the RNA. The hybridization was performed in a dark and humid condition, usually at the temperature of 37°C - 50°C and a hybridization time was from 30 minutes to several hours. After hybridization, the sample was washed in a Washing buffer to remove unbound samples and then the samples were dried and ready for CLSM microscopy.

The strictly specific oligonucleotide probe which was used in this experiment was Lis-1255-Cy3 (specific for *L. monocytogenes* strains). This specific probe was applied in combination with universal (non-specific) probe EUB-338-Fluos which was used for all tested strains of bacteria. The oligonucleotide probes were dissolved in an adequate working solution in concentration of 30ng/μl for fluorochrom-labeled (Cy3) probe and concentration for Fluos probe was 50ng / μl. The probe solution was stored at -20 ° C before use.

The characteristics and specificities of all oligonucleotide probes used in this experiment as well as the percentage of the applied formamide for hybridization buffer are shown in the Table 1. The all probes were synthesized and supplied by Thermo Electron, Division Interactive (Ulm, Germany).

The three-dimensional microscopic analyses of plant roots were done using LSM-510-META (Zeiss, Germany) confocal laser scanning microscopy. The excitation wavelength of He–Ne laser was 543 nm for Cy3 excitation with LP 560 long-pass filter and Cy3 fluorescent dye was shown in red color. The Ar ion laser was excitation wavelength of 488 nm and it was used with BP 500–550 band-pass filter for showing the structure of plant roots.

The enumeration of *L. monocytogenes* cells on the surface and in the interior of plant roots was done by using Zeiss LSM Image Browser software (Zeiss, Germany) as it described by Kljujev et al. (2018) and the number of *L. monocytogenes* cells was calculated and shown as the number of cells per mm3 absolutely dry plant root.

**Results**

**THE PRESENCE OF *LISTERIA* SPP.AND *L. MONOCYTOGENES* IN VEGETABLES ORIGINATED FROM AGRICULTURAL LAND AND GREENGOUSE**

After preliminary tests which were performed on specific selective media for *Listeria* spp., 11 strains were isolated from 11 vegetable samples in total, which were confirmed as *Listeria* spp. The DNA was isolated from all 11 *Listeria* strains and additionally it was isolated DNA from *L. monocytogenes* ATCC19111 which was used as positive control.

The typical *Listeria* spp*.* colonies were 1 - 2 mm in diameter with convex appearance and colorless or opalescent shine on a greenish TSYEA Media. The *Listeria* spp. colonies on Palcam Agar were gray and small (1.5 - 2 mm in diameter) with central depression and black zone around. All isolated strains were Gram positive, asporogene, non-capsular, rod-shaped, catalase positive and motile (showed characteristic umbrella-form growth).

In vegetables sampled in Leskovac location and surroundings, it was not detected neither *Listeria* spp. nor *L. monocytogenes.* The large presence of *Listeria* spp. detected at the Svilajnac location where *Listeria* spp. was detected in most of the vegetable samples (9 samples were positive out of 15 total vegetable samples) (Table 2). As well, at this location, *L. monocytogenes* was isolated from carrot sample (sample number 62) and that is confirmed by PCR analyses with specific primers (Figure 2). This location includes fields with application organic fertilizers but microbiological quality of these fertilizers is unknown and was not analyzed.

According to The Regulation of the Republic Serbia, only samples positive on *L. monocytogenes* are unsatisfactory and unsuitable. The other vegetable samples, originated from organic production, were satisfactory according to the Regulation of the Republic Serbia. Although, the most vegetable samples were positive on *Listeria* spp., there was not presence of *L. monocytogenes*.

At the Obrenovac location, 6 vegetable samples were taken in total from field (2 samples) and plastic greenhouse (4 samples). The samples were taken from two fields which were flooded in 2014. All organically produced vegetable samples were satisfactory according to Regulation and they were negative to *L. monocytogenes* presence. Only one sample (carrot from field) was positive on presence of *Listeria* spp. but it was negative for presence of *L. monocytogenes*. The *L. monocytogenes* was not detected in vegetable samples originated from Gruza-Ljig location, while the only one tested sample was positive to *Listeria* spp. (Table 2).

In general, observing all locations, 25.58% vegetable samples were positive for *Listeria* spp. and only one sample was positive for *L. monocytogenes* out of 43 samples in total, which represents 2.35%.The results showed that vegetable contamination by *Listeria* spp. was the largest in Svilajnac location (60.00% positive samples) and *L. monicytogenes* was detected only in one sample (6.66%). In the Obrenovac location, it was detected 16.66% vegetable samples positive on *Listeria* spp. and in the Gruza-Ljig location it was 11.11% positive samples. The *L. monicytogenes* was detected only on Svilajnac location while this strain was not found in all other locations.

**COLONIZATION VEGETABLE PLANT ROOTS BY *LISTERIA MONOCYTOGENES***

The strain *L. monocytogenes* EGD-E was surface and endophytic colonizer of carrot root but also the high colonization ability was detected at parsley and celery root. In the respect of other examined plants, the significant number of *L. monocytogenes* EGD-E was detected in the surface layers (6.74x105 cells/mm3 absolutely dry root) and inside (1.36x105 cells/mm3 absolutely dry root) of sweet corn root (Figure 3).

On the basis of the obtained micrographs, it was determined that the cells of *L. monocytogenes* EGD-E bind to the surface of the carrot root and they were less commonly found out on root hair. In the inner layers of the root, bacterial cells were inhabited intercellular spaces mainly as single cells (Micrograph 1). At the root of other tested plant species like celery and parsley, bacterial cells were detected on the root hairs. In deeper root layers, bacteria were seen as individual in intercellular spaces and very close to the larval vessels of root (Micrograph 2).

The plant root colonization by *L. monocytogenes* SV4B was the most represented at leafy vegetable plants, lettuce and spinach. The number of bacteria on the root surface of lettuce was 1.68x106 cells/mm3 absolutely dry root and in the root interior, it was detected the less number of cells 4.38x105 cells/mm3 absolutely dry root (Figure 4). On the surface of spinach, it was detected 1.39x106 cells/mm3aps.dry.root of *L. monocytogenes* SV4B and this strain endophyticaly colonized the spinach root in the highest degree compared with other model plants. The very large degree of surface root colonization was also found out at sweet corn. The strain *L. monocytogenes* SV4B showed the smallest ability of colonization of tomato, carrot, parsley and celery roots. The cells of *L. monocytogenes* SV4B strain were visible as single cells in interior tissue of plant roots (celery and sweet corn roots) (Micrograph 3). Also the *L. monocytogenes* cell was visible in the interior of the plant root cell of sweet corn (Micrograph 3 (right)).

**Discussion**

Human pathogen bacteria such as *Shigella* spp., *Salmonella* spp., *Campylobacter* spp., enterohemorrhagic *Escherichia coli*, *Listeria monocytogenes*, *Yersinia enterocolitica*, are the most often isolated strains from fresh vegetables and they indicate the recent fecal contamination (Beuchat, 2002). Some literature data, that point out that human pathogen bacteria are able to build up very close relationship and colonize plant tissues, are focused mainly to *E. coli* (*E. coli* O157: H7) and *Salmonella* spp. (Berger et al., 2010), *Listeria monocytogenes* and *Staphylococcus aureus* (Collignon and Korsten, 2010; Brandl and Mandrell, 2002).

Some authors (Rothballer et al., 2008) experimented with non pathogen bacteria Herbaspirillum frisingense and its ability for colonization *Miscanthus giganteus* and *M. sinensis* plants. Their results showed that bacterial cells were detected inside root cortex three days after inoculation and after one week, the bacterial cells were found in vascular system of plants. These authors worked with GFP-tagged H. frisingense strain and using confocal laser scanning microscopy, they found out bacterial cells in intercellular spaces, cortex and central cylinder of barley roots which means that *H. frisingense* could be an endophytic colonizer of plants.

Our research showed that *L. monocytogenes* EGD-E colonized very intensively carrot root and it was detected 106cells/mm3 aps.dry root at the surface layers of carrot root. Also, the bacterial cells of this strain were detected deeply inside of plant root.

It is known that *Listeria* spp. is cosmopolitan bacteria widespread in the environment and it could be found: in the soil, at plants, in feces of domestic animals, at plants irrigated with contaminated water, etc.

Also, the highest number of *L. monocytogenes* EGD-E cells was detected in the carrot root interior layers (105cells/mm3 aps.dry root) and the smallest degree of colonization was noticed at tomato root. According to some studies, the Listeria infections and outbreaks usually occur during the processing process and manipulation of fresh products (Harvey and Gilmour, 1993). However, there are many reports (Arumugaswamy et al., 1994; MacGowan et al., 1994; De Simon et al., 1992; Vahidy, 1992) that showed the presence of *L. monocytogenes* on cucumber, sweet peppers, potatoes, radishes, leafy vegetables, bean sprouts and seedlings, broccoli, tomatoes, cabbage at the moment of selling these products (point-of-sale). The Farber et al., (1998) showed that *L. monocytogenes* population has a declining trend on carrot for 2-logs in time more than 9 days.

In general, the plant root colonization by *Listeria* spp. strains was the most expressive at root-tuber vegetable plants and according to Heisick et al. (1989a; 1989b) the main reason of colonization is the direct contact between plant tissue and Listeria-contaminated soil. The McLauchlin et al. (2004) consider that a large infectious dose ≈106cells of *L. monocytogenes*, differences in population sensitivity to infection and a long incubation period could explain a lack of proven outbreaks caused by the consumption of Listeria-contaminated fresh products.

Our experimental strain *L. monocytogenes* SV4B showed the best ability for surface and endophytic colonization at leafy vegetables. The smallest degree of colonization by this bacterial strain was noticed at carrot and tomato roots (surface and interior root leyers).

On the contrary of our results, the study of Jablasone et al. (2005) showed that *L. monocytogenes* could not penetrate and enter inside plant tissue of seedlings but this strain had only ability for surface plant colonization and *L. monocytogenes* could survive on the plant surface during the whole cultivation period of the plant. Also, Kutter et al. (2006) investigated the colonization of barley by *L. monocytogenes*, *L. ivanovii* and *L. innocua* using the FISH method with specific oligonucleotide probes for those bacterial strains. They did not detect endophytic colonization but they found out intensive root hairs colonization and they pointed out that this was the main area of plant root colonization by *Listeria* spp. According to them, the barley root colonization by *Listeria* spp. is a rare and sporadic and it occurs with just a few individual bacterial cells. Our results suggest that *L. monocytogenes* is very good endophitic colonizer of vegetable plant roots and it could enter deep in the root, close to xylem system. Also, this bacterium is able to colonize plant root surface and root hairs in high degree.

**Conclusion**

The *L. monocytogenes* is not widespread in nature which reduces infection occurrence, but this bacterium is very good in colonization root of vegetables. Our results showed presence of *L. monocytogenes* in carrot sample and our laboratory experiments confirmed possibility of successful colonization carrot root by this bacterial strain.

The *L. monocytogenes* EGD-E and *L. monocytogenes* SV4Bwere surface and endophytically colonized the root of all investigated plants.

The highest degree of surface and endophytic colonization by *L. monocytogenes* EGD-E was at carrot root. These bacterial cells were the most represented on the surface of carrot root and in the inner root layers. The bacterial cells were inhabited intercellular spaces as single cells. At the root of other tested plant species, *L. monocytogenes* EGD-E cells were detected on the root hairs and in the intercellular spaces very close to the larval vessels of root.

The *L. monocytogenes* SV4B strain colonized lettuce root surface and spinach root interior in the highest degree. The bacterial cells of this strain were visible as single cells in the interior tissue of plant roots. The *L. monocytogenes* SV4B cell was visible in the interior of the sweet corn root cell but the mechanism of transporting bacterial cells inside the plant cell is not clarified yet. Our results suggest that *L. monocytogenes* is very good endophytic colonizer of vegetable plant roots.

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**References**

Amann R. I., Krumholz L., Stahl D. A. (1990) Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J. Bacteriol 172: 762–770.

Arumugaswamy, R. K., G. R. Rahamat Ali, and S. N. B. A. Hamid. 1994. Prevalence of *Listeria monocytogenes* in foods in Malaysia. *Int. J. Food Microbiol.* 23:117–121.

Berger, C. N., Sodha, S. V., Shaw, R. K., Griffin, P. M., Pink, D., Hand, P., Frankel, G. (2010). Fresh fruit and vegetables as vehicles for the transmission of human pathogens. *Environmental Microbiology, 12*, 2385 - 2397.

<http://dx.doi.org/10.1111/j.1462-2920.2010.02297.x>

Beuchat, L. R. 2002. Ecological factors influencing survival and growth of human pathogens on raw fruits and vegetables. *Microbes and Infect.* 4: 413–423.

Blomme, B., and Handler, A. (2009). *Fluorescence In Situ Hybridization*. Retrieved from Enviromental Microbiology:

<http://filebox.vt.edu/users/chagedor/biol_4684/Methods/FISH.html>

Brandl M. T., and R. E. Mandrell. 2002. Fitness *of Salmonella enterica* serovar Thompson in the cilantro phyllosphere. *Appl. Environ. Microbiol.* 68: 3614-3621.

Burun B., Coban Poyrazoglu E. 2002. Embryo Culture in Barley (*Hordeum vulgare* L.). Turk. J. Biol. 26. 175-180.

Centers for Disease Control and Prevention. 2015. List of selected multistate foodborne outbreak investigations. Available at: [http://www.cdc.gov/foodsafety/outbreaks/multistate-outbreaks/outbreakslist.html. Accessed 11 November 2015](http://www.cdc.gov/foodsafety/outbreaks/multistate-outbreaks/outbreakslist.html.%20Accessed%2011%20November%202015).

Collignon S. and Korsten L. (2010): Attachment and Colonization by *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica subsp. Enterica* serovarTyphimurium, and *Staphylococcus aureus* on Stone Fruit Surfaces and Survival througha Simulated Commercial Export Chain. Journal of Food Protection, Vol. 73, No. 7,1247–1256

De Simon, M., C. Tarrago, and M. D. Ferrer. (1992). Incidence of *Listeria monocytogenes* in fresh foods in Barcelona (Spain). *Int. J. Food Microbiol.* 16: 153–156.

Eldor, P. (2007). *Soil Microbiology, Ecology and Biochemistry.* Oxford: Academic Press is an imprint of Elsevier.

Farber J. M., Wang S. L. , Cai Y., Zhang S. (1998). Changes in populations of Listeria monocytogenes inoculated on packaged fresh-cut vegetables. J Food Prot 61(2): 192-5.

Gaul, L. K., N. H. Farag, T. Shim, M. A. Kingsley, B. J. Silk, and E. Hyytia-Trees. 2013. Hospital-acquired listeriosis outbreak caused by contaminated diced celery—Texas, 2010. Clin. Infect. Dis. 56:20–26.

## Golberg D., Kroupitski Y., Belausov E., Pinto R., Sela S. 2011. *Salmonella* Typhimurium internalization is variable in leafy vegetables and fresh herbs. International Journal of Food Microbiology, Volume 145, Issue 1, 31 January 2011, Pages 250-257

Gorski, L.J. D. Palumbo, and R. E. Mandrell. 2003. Attachment of *Listeria monocytogenes* to radish tissue is dependent upon temperature and flagellar motility. *Appl. Environ. Microbiol.* 69: 258-266.

Gorski, L. J. D. Palumbo, and K. D. Nguyen. 2004. Strain-specific differences in the attachment of *Listeria monocytogenes* to alfalfa sprouts. *Food Prot.* 67: 2488-2495.

Hopwood, D. A., Bibb, J. M., Chater, K. F., Kieser, T., Bruton, C. J., Kieser, H. M., Lydiate, K. M., Smith, C. P., Ward, J. M., Schrempf, H. (1985): Genetic manipulation of *Streptomyces*, a laboratory manual. Norwich, UK, The John Innes Foundation.

Kljujev I., Raicevic V., Vujovic B., Rothballer M., Schmid M. (2018) *Salmonella* as an endophytic colonizer of plants - A risk for health safety vegetable production. *Microbial Pathogenesis* 115 (2018) 199–207.

Kutter, S., Hartmann, A. and Schmid, M. (2006). Colonization of barley (*Hordeum vulgare*) with *Salmonella enterica* and *Listeria* spp. **FEMS Microbiology Ecology** 56: 262-271

Harvey J., A. Gilmour (1993): Occurrence and characteristics of *Listeria* in foods produced in Northern Ireland, Int. J. Food Microbiol. 19 (1993) 193–205.

Heisick, J. E., D. E. Wagner, M. L. Neirman, and J. T. Peeler. (1989a). *Listeria* spp. found on fresh market produce. *Appl. Environ. Microbiol.* 55:1925–1927.

Heisick J.E., F.M. Harrell, E.H. Peterson, S. McLaughlin, D.E.Wagner, I.V. Wesley, J. Bryner, (1989b) Comparison of four procedures to detect *Listeria* spp. in foods, J. Food Prot. 52 (1989b) 154–157.

Jablasone, J., Warriner, K., Griffiths, M., (2005) Interactions of *Escherichia coli* O157:H7, *Salmonella* Typhimurium and *Listeria monocytogenes* in plants cultivated in a gnotobiotic system. Int. J. Food Microbiol. 99, 7–18.

Johannessen G. S., Loncarevic S., Kruse H. (2002). Bacteriological analysis of fresh produce in Norway. Int. J. Food Microbiol., 77, 199-204.

MacGowan A.P., K. Bowker, J. McLauchlin, P.M. Bennet, D.S. Reeves (1994) The occurrence and seasonal changes in the isolation of *Listeria* spp. in shop bought food stuffs, human faeces, sewage and soil from urban sources, Int. J. Food Microbiol. 21 (1994) 325–334.

McLauchlin, J., Mitchell, R. T., Smerdon, W. J., Jewell, K. (2004) *Listeria monocytogenes* and listeriosis: a review of hazard characterization for use in microbiological risk assessment of foods. International Journal of Food Microbiology 92, 15–33.

Mengaud, J., Vicente, M. F., Chenevert, J., Pereira, J. M., Geoffrey, C., Gicquel-Sanzey, B., Baquero, F., Perez-Diaz, J.C., Cosart, P. 1988 Expression in Escherichia coli and sequence analysis of the listeriolysin 0 determinant of Listeria monocytogenes. Infection and Immunity 56, 766-772. )

Moter A., and Gobel, U. (2000). Fluorescence in situ hibridization (FISH) for direct visualisation of microorganisms. *Jurnal of Microbiological Methods* , 85-112.

Ozbey G., Hasan Basri Ertas H. B., Kok F. (2006) Prevalence of *Listeria* species in camel sausages from retail markets in Aydin province in Turkey and RAPD analysis of *Listeria monocytogenes* isolates. Irish Veterinary Journal, Volume 59 (6) : 342-344, June, 2006.

Rothballer M., Eckert B., Schmid M., Fekete A., Schloter M., Lehner A., Pollmann S., Hartmann A. (2008) Endophytic root colonization of gramineous plants by *Herbaspirillum frisingense*. FEMS Microbiology Ecology, Volume 66, Issue 1, October 2008, Pages 85–95.

Shenoy A., Oliver H., Deering A. 2017. *Listeria monocytogenes* Internalizes in Romaine Lettuce Grown in Greenhouse Conditions. Journal of Food Protection, Vol. 80, No. 4, 2017, Pages 573–581

Swaminathan, B. (2001) *Listeria monocytogenes*. In *Food microbiology: fundamentals and frontiers.* (Eds. M. P. Doyle, L. R. Beuchat, & T. J. Montville)*,* Washington, DC,ASM Press, pp. 383-409.

### The Official Journal of Republic of Serbia No. 72/2010. Regulations of general and special conditions for food hygiene at any stage of production, processing and trade. Serbia, Belgrade, 2010 (*in Serbian: Pravilnik o opštim i posebnim uslovima higijene hrane u bilo kojoj fazi proizvodnje, prerade i prometa. Sl.glasnik RS br. 72/2010*).

Thunberg R., Train T., Bennett R., Matthews R., Belay A. 2002. Microbial Evaluation of Selected Fresh Produce Obtained at Retail Markets. Journal of Food Protection, Vol. 65, No. 4, 2002, Pages 677 – 682.

Vahidy R., (1992) Isolation of *Listeria monocytogenes* from fresh fruits and vegetables, Abstract, HortScience 27 (1992) 628–628.

**Table list:**

Table 1. The characteristics and specificity of oligonucleotide probe used in experiment

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Bacteria** | **Probe** | **Specificity** | **Binding position** | | **Sequence 50-30** | **% FA** | **Reference** |
| **Target**  **(rRNA)** | ***E. coli*** |
| *L. monocytogenes* | Lis-1255 | *Listeria sp.*  *B. thermosphacta*  *B. campestris* | 16 S | 1255-1272 | ACCTCGCGGCTTCGCGAC | 35 | Wagner et  al. (1998) |
| All bacteria | EUB-338 | *Bacteria* | 16 S | 338-355 | GCTGCCTCCCGTAGGAGT | 35 | Amann et al. (1990) |

Table 2. The presence of *Listeria* spp. and *L. monocytogenes* in vegetables.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Location – Leskovac (1) | | | |
| Vegetable | ***Location*** | ***Way of crop cultivation*** | ***Listeria* spp.** | ***L. monocytogenes*** |
| *Tomato* | 1.1. | Plastic greenhouse 1 | - | - |
| *Sweet peppers* | 1.1. | Plastic greenhouse 2 | - | - |
| *Tomato* | 1.2. | Plastic greenhouse | - | - |
| *Sweet peppers* | 1.2. | Plastic greenhouse | - | - |
| *Tomato* | 1.3. | Plastic greenhouse | - | - |
| *Sweet peppers* | 1.3. | Plastic greenhouse | - | - |
| *Cabbage* | 1.3. | Field | - | - |
| *Sweet peppers* | 1.2. | Plastic greenhouse | - | - |
| *Tomato* | 1.2. | Plastic greenhouse | - | - |
| *Sweet peppers* | 1.4. | Plastic greenhouse | - | - |
| *Tomato* | 1.4. | Plastic greenhouse | - | - |
| *Sweet peppers* | 1.4 | Field | - | - |
| *Hot peppers* | 1.5. | Plastic greenhouse | - | - |
| Location – Svilajnac (2) | | | | |
| *Tomato* | 2.1. | Greenhouse | - | - |
| *Sweet peppers* | 2.1. | Greenhouse | - | - |
| *Cucumber* | 2.1. | Greenhouse | - | - |
| *Potato* | 2.2. | Field | + | - |
| *Tomato* | 2 | Plastic greenhouse | - | - |
| *Sweet peppers* | 2 | Plastic greenhouse | **+** | **-** |
| *Carrot* | 2 | Field | + | + |
| *Parsley* | 2 | Field | + | - |
| *Potato* | 2.3. | Field | + | - |
| *Tomato* | 2.3. | Field | - | - |
| *Carrot* | 2.3. | Field | + | - |
| *Tomato* | 2.4. | Plastic greenhouse | - | - |
| *Sweet peppers* | 2.4. | Plastic greenhouse | + | - |
| *Carrot* | 2.4. | Field | + | - |
| *Potato* | 2.4. | Field | + | - |
| Location – Obrenovac (3) | | | | |
| *Tomato* | 3.1. | Plastic greenhouse | - | - |
| *Sweet peppers* | 3.1. | Field | - | - |
| *Carrot* | 3.2. | Field | + | - |
| *Sweet peppers* | 3.2. | Plastic greenhouse | - | - |
| *Tomato* | 3.2. | Plastic greenhouse | - | - |
| *Tomato* | 3.3. | Plastic greenhouse | - | - |
| Location – Gruza-Ljig (4) | | | | |
| *Potato* | 4.1. | Field | - | - |
| *Cabbage* | 4.2. | Field | - | - |
| *Tomato* | 4.3. | Field | - | - |
| *Sweet peppers* | 4.4. | Field | - | - |
| *Potato* | 4.5. | Field | + | - |
| *Sweet peppers* | 4.6. | Plastic greenhouse | - | - |
| *Tomato* | 4.7. | Plastic greenhouse | - | - |
| *Tomato* | 4.7. | Plastic greenhouse | - | - |
| *Tomato* | 4.8. | Plastic greenhouse | - | - |

**Figure list:**



Figure 1. Vegetable sampling locations with labeled cities 1 – Leskovac; 2 – Svilajnac; 3 – Obrenovac; 4 – Gruža-Ljig and their rural surroundings presented in sizable windows.

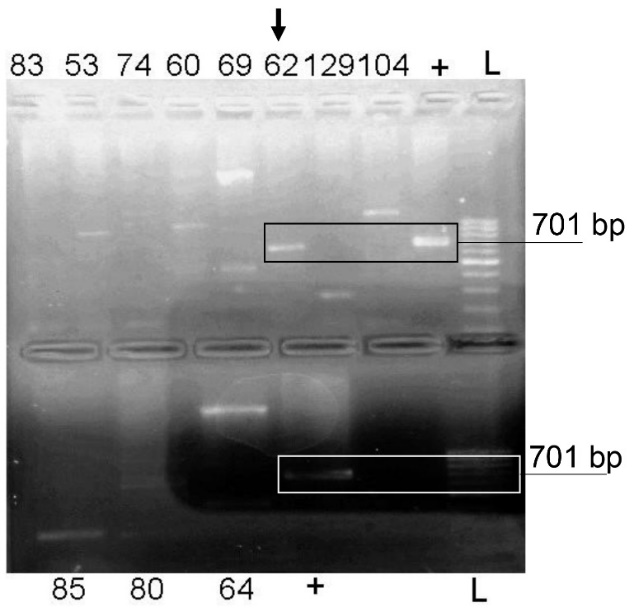
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Figure 2. An agarose gel with PCR amplification of the 701 bp fragment on listeriolysin O sequence (hlyA) gene for identification *L. monocytogenes* isolated from vegetable samples (line L - 100 bp DNA ladder; line + - positive control; lines 83, 53, 74, 60, 69, 129, 104, 85, 80, 64 – *Listeria* isolates; line 62 - *L. monocytogenes* isolate)

Figure 3.Colonization plant roots by *Listeria monocytogenes* EGD-E

( Root surface; Root interior)

Figure 4. Colonization plant roots by *Listeria monocytogenes* SV4B

( Root surface; Root interior)

**Micrograph list:**



Micrograph 1. Colonization carrot root by *Listeria monocytogenes* EGD-E. The bacterial cells are visible in yellow color and they are located in intercellular spaces inside plant root.

Micrograph 2.Colonization celery root (left) and parsley root (right) by *Listeria monocytogenes* EGD-E. The bacterial cells are visible in yellow color

Micrograph 3. Colonization celery root (left) and sweet corn root (right) by *Listeria monocytogenes* SV4B. The bacterial cells are visible in yellow color. The white arrows show single bacterial cells inside plant root tissue.