

PRESENCE OF *LISTERIA* SPP IN FISH SAMPLES, FISH PRODUCTS AND SEA PRODUCTS

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Listeria monocytogenes is a bacterium that is pathogenic for man and for most animal species. Listeriosis is a generalized infection that starts after ingestion of the causative agent *L. monocytogenes* (Finlay, 2001). Food that is not properly thermally processed, long storage of such food, food that is produced in inadequate hygienic food plants, as well as cooked-cooled ready to eat food (RTE) is the ideal medium for listeria multiplication. High risk food originated from fish, fish products and sea products comprised of: molluscs (fresh or frozen shellfish, crustaceans shelled or not), fresh fish (ready to eat without cooking), fish products packed with brine (NaCl<6%), (salted, marinated, fermented, cold smoked and fish in brine), thermally treated fish and crustacean products (pasteurization, cooking, hot smoking, including pre-cooking and panning).

In this research, fish samples, fish products and sea products from Serbian markets were examined such as: fresh fish (cooled), frozen food (fish and sea products – cuttlefish, squid, octopus, shellfish, crustaceans and shrimps), panned products; smoked fish, salted fish, thermally treated fish and fish products, semi-canned fish and canned fish. Microbiological testing has been performed according to internationally prescribed standards ISO 11290-1 (1996) "Microbiology of food and animal feed stuffs – Horizontal method for the detection and enumeration of *Listeria monocytogenes*". There were 470 samples from fish products and sea products. Presence of *Listeria* spp was registered in 58 samples (12.34%). *Listeria monocytogenes* was found in 1.92% samples (9 isolates), which makes 15.52% of the total *Listeria* that were isolated from the tested food. Other isolated *Listeria* belong to the following species: *L. innocua* (8.51%), *L. welshimeri* (1.28%), *L. welshimeri/innocua* (0.21%), *L. grayi* (0.21%) and *L. seeligeri* (0.21%). Exceptional viability of *Listeria monocytogenes* in the food tested was documented, also (freezing temperature – 18°C, five months).

Key words: *Listeria monocytogenes*, fish, sea products

INTRODUCTION

Listeria monocytogenes is a causative agent of human listeriosis. Food (apart of milk and dairy products, fish, fish products and sea products) is frequently a vector of human listeriosis. The reason why listeriosis is a complex disease is that this bacteria has the ability to initiate self-phagocytosis ("loading into the host cells"). *Listeria* multiply in the cell cytoplasm and transport to other cells (Kuhn and Goebel, 1999). *Listeria* enter perorally, enter the enterocytes, as well as M cells, in the vicinity of Payer's patches. At this time, multiplication of bacteria starts in phagocytic cells that are beneath the enterocytes (Decatur and Portnoy, 2000). The next step is the transport of *Listeria* hidden in macrophages into the blood and lymph, finally reaching the liver and spleen. The vast majority of *Listeria* is destroyed by neutrophils in the vicinity of Kupfer cells. However, if cellular immunity of the host is compromised or inadequate, *Listeria* can multiply in the hepatocytes, as well as in the macrophages. In such a case, bacteria can be transported by the blood to other organs, particularly the brain and/or placenta. Bacteria can pass the brain barrier and the placenta. During each of the phases in *Listeria monocytogenes* pathogenesis, bacteria synthesize several virulence factors: internal (enabling *Listeria* penetration in non-phagocyte cells: epithelial cells, hepatocytes); superficial protein p104 (bacterial adhesion to the intestinal cells); Listeriolysin O (cytoplasm vacuola membrane lysis, hence enabling the bacteria to escape into the cytoplasm of the host cell); ActA protein (polymerization of the globular actin molecules in order to form the actin tail that enables bacteria to move to the cell membrane where *Listeria* form listeriopods that attack neighbouring cells (Moors *et al.*, 1999). In such a way, *Listeria* spread locally, with no possibility to get into contact with anti-*Listeria* antibodies and other immunoreactive molecules; phospholipases, metalloproteases, C1p proteases, and ATP-ases; protein p60 (Dons *et al.*, 1999; Jin *et al.*, 2001).

Listeria monocytogenes is an opportunistic ubiquitous bacteria. That means that it is widely distributed in the environment. So, there is a possibility for it to be present not only in diseased, but in healthy animals, as well. Moreover, *Listeria* can cause food contamination during production and storage. That is how food becomes a source of infection for man and animals.

Apart of *Listeria monocytogenes*, there are other bacteria that can be a source for human infection after consumption of contaminated food: *Campylobacter*, *Salmonella*, *Yersinia* and pathogenic strains of *Escherichia coli*. Such bacteria cause well known food borne diseases (Schlundt, 2002).

Listeria monocytogenes is a pathogen for man and many animal species. Most often, it results in sporadic infections, however it can cause epidemics, as well.

The most frequent clinical sign is meningitis followed by septicaemia. During pregnancy, listeriosis is most frequent in the third trimester of intrauterine life. Infection of the pregnant female can be without clinical signs or only with clinical signs of mild influenza. Infection of the central nervous system during pregnancy is exceptionally rare. However, the consequences for the foetus can be serious: miscarriage, foetal death, preterm delivery and other symptoms of

neonatal septicaemia, as well as meningitis (Frederiksen and Samuelsson, 1992). The first case of listeria epidemics was recorded by Nyfeldt (1929). However, it was only after 1981 that food was defined as a source of listeriosis in the human population. Schillech *et al.* (1983) found that listeriosis can be caused by lettuce salad. Two years later Fleming showed that full fat and skimmed (2% fat) milk (Fleming *et al.*, 1985) can be the source of infection and Linnan (Linnan *et al.*, 1988) showed that soft Mexican cheese samples were contaminated with *Listeria monocytogenes*. On the basis of these data, Schuchat *et al.* (1992) and Pinner *et al.* (1992) concluded that food is a major vector for human listeriosis. The Californian epidemic of listeriosis in 1985, numbering a total of 142 infected patients with 48 fatal cases, was the last and major strike needed to define *Listeria monocytogenes* as an important pathogen for man (ICMSF, 1996).

Vegetables, meat and milk products, fish and sea products can be contaminated with *Listeria* spp. Such food has been pointed out as a source of listeriosis in humans (Rorvik *et al.*, 2000). Apart of that, listeria have the unique possibility to multiply at 4 °C which is the common refrigerator temperature (Hof *et al.*, 2000). *Listeria monocytogenes* can multiply in a wide pH range, wide temperature range, as well as low water activity. At 4 °C the generation time for *Listeria monocytogenes* is 12 to 36 hours. If food is stored for a long time, at +4 °C the bacteria number can increase up to 10⁶/gram. *Listeria monocytogenes* is halo tolerant and survives up to 100 days at 30.5% salt concentration at +4 °C.

The best environment for listeria growth is thermally untreated food, food that spent a long time during storage, food that has been produced in non-hygienic food plants as well as cooked-cooled meals "ready to eat" (RTE).

Listeria monocytogenes has been isolated from river water and river sediment (Colburn *et al.*, 1990), channels, ponds and lakes (Dijkstra, 1982), as well as from sea water and water shorelines (Colburn *et al.*, 1990). Because of that, it can be expected that *Listeria monocytogenes* can be present in water organisms (Karunasagar and Karunasagar, 2000). Data accumulated during the last decade show that there is food with different and various risk levels as listeriosis vectors (Rocourt *et al.*, 2000).

Among fish, fish products and sea products, there is some high risk food such as: molluscs (fresh and frozen shellfish, crustaceans – in the shell or deshelled), fresh fish (that is intended to be consumed without thermal treatment), fish products in brine (NaCl6%) in water (salted, marinated, fermented, cold smoked fish and fish in self sauce), mid-heat treated fish and crustacean products (pasteurization, cooking, hot smoking including pre-cooking and panning).

MATERIAL AND METHODS

In this research, fish, fish products such as fresh (cooled) fish; frozen food (fish, sea products – cuttlefish, squid, octopus, shellfish, crustacean and shrimps), panned products; smoked fish; salted fish, heat treated fish and fish products, semi-canned fish products, as well as canned fish were examined.

Isolation and identification of Listeria monocytogenes

Microbiological tests have been performed according to international standards: ISO 11290 – 1 (1996) "Microbiology of food and animal feeding stuffs - Horizontal method for the detection and enumeration of *Listeria monocytogenes*". Samples (25 gram) were homogenized with 225 mL of half Fraser broth (liquid media) in a sterile sample bag in a stomacher for 30 seconds. Thereon the sample was incubated at 30 °C for 24 hours. After incubation, 0.1 mL of the sample was inoculated into 10 mL of Fraser liquid media which was subsequently incubated 48 + 2 hours (35 - 37 °C). The material from half Fraser liquid media was inoculated into solid Palcam, as well as Oxford agar media and incubated 24 - 48 hours (30 °C and 35 - 37 °C). From the Fraser liquid media, after incubation the sample was transferred on to Palcam and Oxford solid agar media.

Colonies of the microorganism on Palcam agar after 24 hours of incubation, that show the characteristic listeria growth morphology: small (1.5 – 2.0 mm), gray-green or olive colour, sometimes with a central black zone and possess the typical halo appearance. After 48 hours of incubation, colonies are larger, green with a central depression and with a halo appearance. *Listeria* colonies on Oxford agar have a typical appearance: after 24 hours of incubation, colonies are small (1.0 mm in diameter), greyish, encircled with a black zone. After 48 hours of incubation, colonies are slightly larger (2.0 mm), darker, with a greenish reflection and with a black halo appearance. Typical colonies were transferred on to TSEYA agar and incubated for 18 – 24 hours (35 - 37 °C).

Characteristic colonies were picked out and transferred on to ordinary nutrient agar and blood agar in order to perform catalase test and in order to prepare samples for Gram staining.

In order to obtain the pure bacterial culture, as well as to register any sign of haemolysis the blood agar was incubated for 24 hours (37°C). Instant appearance of bubbles was the criterion for positive test results. Gram positive, catalase positive, oxidase negative colonies that grow in the umbrella appearance in mobility solid media (at 35 °C), assume as a characteristic morphology for *Listeria* spp. *Listeria* species differentiation is performed according to the CAMP phenomenon along with beta-haemolytic *Staphylococcus aureus* strain and with *Rhodococcus equi*, as well as by using API *Listeria* (bioMerieux) test.

RESULTS AND DISCUSSION

Out of the 470 samples tested, 58 were listeria positive (12.34%). One sample was positive out of 43 fresh fish samples. Out of the 161 tested samples of frozen fish, 9 were positive. Twenty seven samples were positive out of 43 samples of panned fish products. Out of the 72 samples of smoked fish 6 were positive. One sample was positive out of tested 15 samples of salted fish. In total 10 samples of sea products were positive out of the 81 tested (Table 1). Laciari and Centorbi (2002) tested one hundred samples (fish, squid, molluscs and shellfish) along the Argentinian coast of the Atlantic ocean. They found that 12 samples (12%) were contaminated with *Listeria* spp.

Table 1. Isolation and *Listeria* spp present in fish, fish products and sea products

Sample	Number of samples tested	Number of <i>Listeria</i> spp positive	Percent of <i>Listeria</i> spp found in sample
Fresh fish	43	1	2.33
Frozen fish	161	9	5.59
Panned fish products	43	27	62.80
Smoked fish	72	6	8.33
Salted fish	15	1	6.67
Half – cans (fish)	15	–	0
Fish cans	20	–	0
Heat treated products and products ready to eat fish food	20	4	20
Sea products	81	10	12.35
Total	470	58	12.34

Out of the total number (43) of fresh fish samples, 37 were sea fish and 6 were fresh water fish. Only one sample of fresh water fish (bighead carp), chilled and chopped was *Listeria innocua* positive (Figure 1).

No sample of fresh sea fish (37) was *Listeria* positive. This finding is especially important since no sample was contaminated with pathogenic *Listeria monocytogenes*. According to literature data (Dhanashree *et al.*, 2003), *L. innocua* can be present in 3.8% of such samples while *L. monocytogenes* can contaminate 1.3% of the fresh fish samples. No other species of *Listeria* spp has been isolated. *L. monocytogenes* was isolated from 4.2 % of fresh molluscs (serovariety 1) and 2.9% from fresh sole fish serovariety 4 (Inoue *et al.*, 2000). No sample taken from smoked fish was contaminated with listeria (Dhanashree *et al.*, 2003).

Out of 161 samples of tested frozen fish, 9 were *Listeria* species positive (5.59%) (Figure 2).

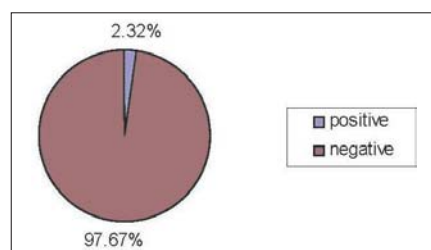


Figure 1. Fresh fish samples-presence of *Listeria* spp.

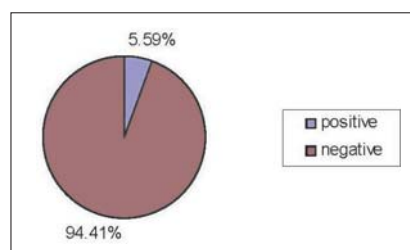


Figure 2. Samples of frozen fish-presence of *Listeria* spp.

Testing all 16 samples of fresh water fish (10 samples of California trout and 6 carp samples) none was *Listeria* spp positive. Out of 9 *Listeria* species that were isolated, four of them belong to *Listeria monocytogenes*, 2 of them were *Listeria innocua*, 2 of them were *Listeria welshimeri* and one was *Listeria seeligeri* species. Forty five samples of frozen hake were tested and 3 of them were positive on *Listeria* species, 2 of them (4.44%) were pathogenic *Listeria monocytogenes* positive and one sample (2.22%) was *Listeria innocua* positive.

Forty three samples of panned fish products were tested (Figure 3).

In these samples, an exceptionally high percentage of *Listeria* positive was found. Out of the total number of samples (43), 27 (62.8%) were *Listeria* positive. Out of 27 isolated *Listeria* spp from panned fish products, 20 were identified as *Listeria innocua* (74 %). Four isolates belong to *Listeria welshimeri* (14.8%). Three isolates were determined as *Listeria monocytogenes*, *L. grayi* and *L. welshimeri/innocua*, each.

Seventy two smoked fish samples were tested (Figure 4).

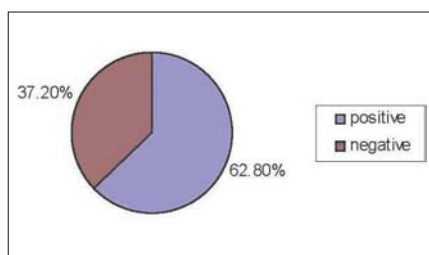


Figure 3. Samples of panned fish products-presence of *Listeria* spp.

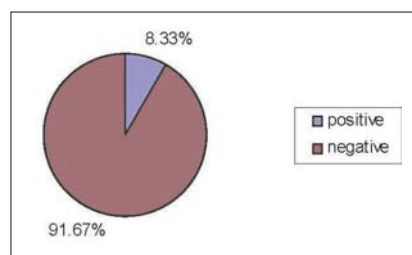


Figure 4. Smoked fish samples-presence of *Listeria* spp.

There were only six listeria positive (8.33%), in the following samples: smoked salmon, smoked herring and smoked trout fillet. Two isolates belong to *Listeria monocytogenes* and four isolates were *L. innocua*. Other *Listeria* spp were not isolated from smoked fish.

Dillon *et al.* (1994) tested the presence of *Listeria* spp in samples of smoked fish products on the market in Newfoundland. Out of 116 cold smoked products, 7 of them were listeria contaminated (6.03%). Out of 142 samples of hot smoked products, 26 were *Listeria* positive (18.3%). Total percentage of isolated listeria was 16.7%. They found that cold samples were the most contaminated ones (46.7%). Eighteen samples were *L. innocua*, 13 of them were *L. welshimeri* and 12 of them were *Listeria monocytogenes*.

Fifteen samples of salted fish were tested (Figure 5).

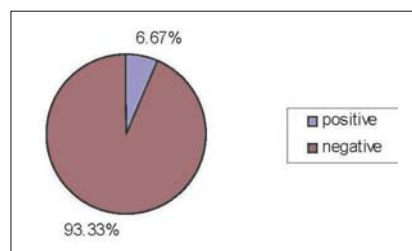


Figure 5. Samples of salted fish-presence of *Listeria* spp.

In one (6.67%) sample of salted anchovy listeria was detected. Subsequently, this sample was defined as a *Listeria innocua* isolate. Presence of *Listeria* spp was not demonstrated neither in 20 fish cans (sterilized products) nor in 15 semi-canned (pasteurized products). In twenty samples from heat treated products, *Listeria* spp were found in 4 (20%) samples. All samples were contaminated with *Listeria innocua*.

Eighty one sample was tested on the presence of *Listeria* species in sea products. Out of them, there were 61 frozen samples, 3 fresh samples, 5 of them were canned sea products, heat treated ready to use were eight samples and 4 canned sea products samples. Among other fresh sea products tested, there were 3 shellfish samples: oysters, mussels, and date shells, and these samples were chilled and kept on ice until use. Neither sample of shellfish was listeria contaminated.

Sixty one sample of frozen sea products were tested. Six of them (9.84%) were *Listeria* spp contaminated. Two isolates were *Listeria monocytogenes* and four of them were *Listeria innocua*. Five canned sea products were tested. Two of them were contaminated with *L. innocua*.

Eight samples of ready to use sea products that were already heat treated were tested. In two of them, there was listeria contamination. In those samples, *L. innocua* was isolated. Four canned sea products were tested as well (mussels, mussels in vinegar, squid, squid in sauce). No one of the canned sea product was listeria contaminated.

Testing of the all sea products samples revealed that out of 81 samples, 10 (12.35 %) of them were *Listeria* spp contaminated. Two isolates (2.47%) were *Listeria monocytogenes* and 8 of them (9.88 %) were *L. innocua*. Other listeria species were not isolated in the tested sea products.

Already published data (Karunasagar and Karunasagar, 2000) show that *Listeria monocytogenes* can contaminate tropical sea fish as follows: 2% in heat treated fish, 17.2% in frozen molluscs, 12.1% in edible frozen shellfish, 1.5% in fresh non treated molluscs and in 4% in fresh non treated edible shellfish.

Fifty eight samples, out of a total of 470 samples were *Listeria* spp positive (Figure 6).

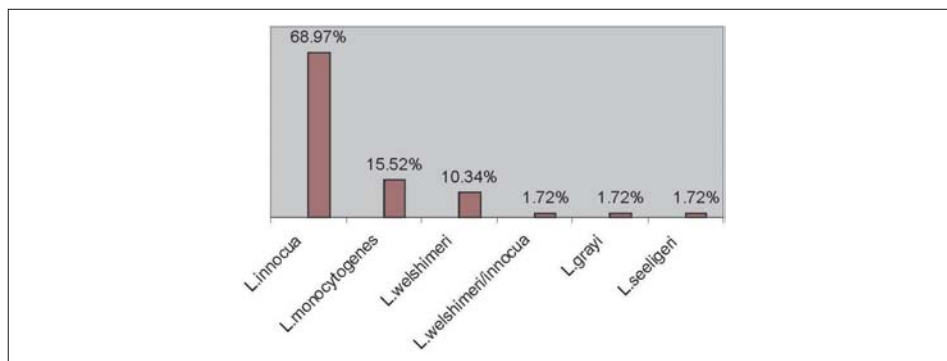


Figure 6. Percent of *Listeria* spp found in all tested samples

Isolated bacteria were as follows: *L. monocytogenes* – 9 isolates (15.52%), *L. innocua* – 40 isolates (68.97%), *L. welshimeri* – 6 isolates (10.34%), *L. welshimeri/innocua* – 1 isolate (1.72%), *L. grayi* – 1 isolate (1.72 %) and *L. seeligeri* – 1 isolate (1.72 %).

Listeria monocytogenes was isolated from 4 frozen fish samples, from one panned fish products sample, from 2 smoked fish samples and from 2 frozen sea product samples.

Out of a total of 470 samples of fish, fish products and sea products, 9 of them were *Listeria monocytogenes* positive. That means that 1.92% of all samples were contaminated with *L. monocytogenes*.

Embarek (1994) tested fish products for *Listeria monocytogenes* contamination on a global level. He published that there was a listeria contamination at a range from 4 to 12%. Moreover, he concluded that contamination depends on temperature. Laciari and Centorbi, (2002) found that *Listeria* spp contamination was 12 percent. Out of all isolated species, three of them were *Listeria monocytogenes*. The bacteria was isolated from different molluscs species. Other nine isolates were *Listeria innocua*.

It is interesting to point out that all samples before testing were stored for several (4 – 5) months at – 18 °C. In spite of that, bacteria that contaminated the tested samples survived. After the thawing process bacteria were still able to multiply. This is important data that supports a well known listeria feature: high resistance of *Listeria monocytogenes* at cold regime.

Listeria innocua was the most frequently isolated *Listeria* species. It was isolated from forty isolates as follows: fresh fish – one isolate, frozen fish – two isolates, panned fish products – twenty isolates, smoked fish – four isolates, salted fish – one isolate, heat treated products ready for use – four isolates, frozen sea products – four isolates, panned products – two isolates, heat treated and ready for use – two isolates.

Listeria welshimeri was isolated from six samples: frozen fish – two samples and panned fish products, four isolates. *Listeria welshimeri/innocua* species that was identified, share mutual characteristics that are typical for both listeria species. This bacteria was isolated from one panned fish sample. *Listeria grayi* was isolated from one panned fish product sample. *Listeria seeligeri* was isolated from one frozen fish sample.

On the basis of the abovementioned data, it can be concluded that the most frequent listeria species that was present in tested samples was *Listeria innocua* (68.97%). Its presence was most frequent in panned fish products (74%). However, this listeria species is not considered to be pathogenic neither for man or for animals.

Listeria monocytogenes as a pathogenic listeria species, was at the second place, as far as isolation frequency from tested samples from fish, fish products and sea products, was concerned. This species was isolated from 15.52% of positive samples. The most frequent isolation of this pathogenic bacteria was from frozen food (four frozen fish and two samples of sea products): 66.66% of all isolates. However, since this has to be heat treated before consumption the probability to cause alimentary infections is diminished. Danger of alimentary

infection in man is if such products (fish meat and fish products) are thermally sub-treated. Pathogenic *Listeria monocytogenes* was also isolated from one canned fish product and from two samples of smoked fish. That is why such products should be properly treated (using high temperatures) in order to achieve temperature required for listeria to be inactivated.

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PRISUSTVO LISTERIJA VRSTA U UZORCIMA RIBA, PROIZVODA OD RIBE I MORSKIH PLODOVA

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SADRŽAJ

Listeria monocytogenes je bakterija patogena za ljude i veliki broj životinjskih vrsta. Listerioza predstavlja generalizovanu infekciju koja nastaje nakon oralne ingestije uzročnog agensa *L. monocytogenes* (Finlay, 2001). Navedena bakterija se najbolje razvija u namirnicama koje nisu adekvatno termički obrađene; koje su dugo skladištene; koje su proizvedene na mestu na kojem principu higijenskog rukovanja hranom nisu sprovedeni; kuvano-hladnoj hrani spremnoj za jelo (ready to eat food - RTE)

U visoko rizične namirnice počev od vrsta ribe, proizvoda od ribe i plodova mora ubrajaju se: mekušci (sveže i zamrznute školjke, rakovi u ljušturama, ili očišćeni), sveža riba (koja se konzumira bez termičke obrade), riblji proizvodi u vodenom rastvoru soli (NaCl%), (soljena, marinirana, fermentisana, hladno dimljena i riba u sopstvenom soku), srednje termički obrađeni riblji proizvodi i rakovi (pasterizacija, kuvanje, toplo dimljenje, uključujući i pred-kuvanje i panirani proizvodi).

Kao materijal za ispitivanje korišćeni su uzorci riba, proizvoda od riba i morski plodovi: sveža riba (u rashlađenom stanju), zamrznute namirnice (riba i morski plodovi - lignje, sipe, hobotnice, školjke, rakovi i škampi), panirani proizvodi, dimljena riba, usoljena riba, termički obrađena riba i proizvodi od ribe, polukonzerve i konzerve od ribe. Mikrobiološko ispitivanje je rađeno prema propisanoj me-

todi po Međunarodnom standardu ISO 11290-1 (1996) „Microbiology of food and animal feeding stuffs - Horizontal method for the detection and enumeration of *Listeria monocytogenes*“. Ukupno je ispitano 470 uzoraka ribe, proizvoda od ribe i morskih plodova. Prisustvo *Listeria* vrsta utvrđeno je u 58 uzoraka (12,34%). *Listeria monocytogenes* je utvrđena kod 1,92% pregledanih uzoraka (9 izolata) i čini 15,52% od svih vrsta iz roda *Listeria* koje su izolovane iz navedenih namirnica. Ostale vrste listerija koje su izolovane pripadale su: *L. innocua* (8,51%), *L. welshimeri* (1,28%), *L. welshimeri/innocua* (0,21%), *L. grayi* (0,21%) i *L. seeligeri* (0,21%). Ustanovljena je izuzetna sposobnost preživljavanja pri niskim temperaturama vrste *Listeria monocytogenes* u namirnicama koje su čuvane pri temperaturi od -18°C tokom 5 meseci.

