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LISTERIA MONOCYTOGENES BACTERIUM IN THE AREA OF MOSTAR: OCCURRENCE IN VEGETABLES AND HEALTH RISKS

Mirza Kovačić[1]; Aida Šukalić[1]; Kristina Doko[2]; Vedrana Komlen[1]; Alma Memić[1]
[1]University "Džemal Bijedić", Agromediterraneane Faculty
kova.mo@hotmail.com; aida.sukalic@unmo.ba; vedrana.komlen@unmo.ba; alma.memic@unmo.ba
[2]Federal Agro-Mediterranean Institute Mostar, kristina.doko@faz.gov.ba

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Abstract:

This research focuses on the bacterium *Listeria monocytogenes* and its occurrence in vegetables, specifically, tomatoes and peppers, and the risks this bacterium poses to health. The research included laboratory analysis of 16 samples of tomatoes and peppers, as well as survey research in the area of the City of Mostar. Based on the survey questionnaire, the respondents showed their knowledge about the occurrence of the bacteria *Listeria monocytogenes* in food and their general knowledge about listeriosis. Laboratory analysis consists of two parts. The first part refers to the laboratory analysis of the samples conducted in the Federal Agro-Mediterranean Institute laboratory in collaboration with its employees. The second part refers to an additional laboratory analysis conducted in the Veterinary Institute of Herzegovina–Neretva Canton laboratory in Mostar. This laboratory research aimed to establish whether the bacterium *Listeria monocytogenes* was present in the samples of vegetables examined in the analysis.

All the vegetable samples were chosen randomly from four different locations in the area of Mostar. Also, the analysis included the examination of the washed and unwashed tomatoes and peppers.

After conducting the laboratory research, it was established that the bacterium *Listeria monocytogenes* was not found in the samples used, which means all the examination results were negative.

Keywords: *Listeria monocytogenes*, peppers, tomatoes, laboratory analysis

1. Introduction

Listeria monocytogenes, a pathogenic bacterium ubiquitous in the natural environment, is the cause of listeriosis in humans. Listeriosis in humans is not such a common disease. However, it generally requires hospitalization of patients, and death cases are not rare. Food contaminated with *L. monocytogenes* is the cause of a large number of cases of listeriosis in humans, so it is crucial to ensure its multi-level control in all stages of production.

The control of *L. monocytogenes* along the entire food chain is necessary to achieve microbiological food safety. It starts from primary production (farm animals and their food) and the production of plants and seeds to planting and distribution (retail and catering). Food safety criteria in the entire chain are defined by the Regulation of the European Commission (Regulation [EC] No. 2073/2005), which has been transferred entirely into the legislative acts of the member countries, as well as countries that are in the process of joining the European Union.

The infection, more precisely listeriosis caused by *Listeria monocytogenes*, is a rare but dangerous disease for susceptible individuals. Even with adequate antimicrobial treatment, it has a mortality rate of 20-30 percent (Osimani and Clementi 2016).

According to Commission Regulation (EC) No. 2073/2005, on microbiological criteria for food, *Listeria monocytogenes* bacterium must not be present in a number greater than 100 cfu/g (colony forming units) during

[1] University of Dzemal Bijedic in Mostar, Faculty of Agromediterranean
[2] Federal Agro-Mediterranean Institute Mostar

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the declared shelf life of the product. Also, the bacterium *Listeria monocytogenes* must not be present in 25 g of the product being placed on the market.

L. monocytogenes is found in fresh vegetables. The increase in the contamination of fresh vegetables in "fast-food" restaurants is considered to be the main reason for human infection (Berrada 2006).

This pathogen was found and isolated from a wide range of samples of different types of food (cheese, fermented meat, ice cream, raw milk, raw and cooked meat, raw vegetables, and smoked seafood), wastewater, silage, soil, water, etc. For a long time, this bacterium has been recognized as the cause of certain diseases in humans. However, it was only in the 1980s that it was noticed that contaminated food was the primary way of transferring this bacterium from the environment to humans (Magdalenić B., 1993).

In 2004, the World Health Organization (WHO) published a technical report on the risk assessment of *L. monocytogenes* in ready-to-eat (RTE) foods in response to a request by the Codex Committee on Food Hygiene (CCFH) for scientific proposals as a basis for the development of guidelines for the control of *L. monocytogenes* in food (WHO, 2004).

According to data from EFSA (European Food Safety Authority), in 2017, 2 480 cases of human listeriosis were reported in 28 countries of the European Union, of which 227 resulted in death. A statistically significant growth of confirmed cases of listeriosis in EU countries from 2008 to 2017 was shown (EFSA and ECDC, 2018).

The primary causes of *Listeria monocytogenes'* occurrence are the poor microbiological quality of raw food, contamination, cross-contamination, inadequate cleaning and preparation process, unsafe storage temperature, and lack of staff training in food hygiene (Osimani and Clementi, 2016).

This research aimed to determine the presence of the bacterium *Listeria monocytogenes* in fresh vegetables, tomatoes and peppers, in the area of the City of Mostar. Then, based on a questionnaire, to determine whether and to what extent residents are familiar with this pathogen, the occurrence of listeriosis, and whether hygienic practices are respected to produce a safe product for consumption.

2. Material and Methods

Given that tomatoes and peppers are the vegetables most consumed during the summer, i.e. in their season, they were selected for the research. Eight tomato samples (four washed and four unwashed) and eight pepper samples (four washed and four unwashed) were collected from the market in the City of Mostar. The samples were purchased at Stara Tepa Mostar, Gradska tržnica Mostar, Brojler supermarket, and MFM store.

Microbiological analysis for the presence of *Listeria monocytogenes* was performed in the microbiological laboratory of the Federal Agro-Mediterranean Institute in Buna. The following analyses were performed:

- Horizontal method for the detection and enumeration of *Listeria monocytogenes* - Part 1: Detection Method BAS EN ISO 11290-1:2005
- *L. monocytogenes* detection using ALOA plating medium
- *L. monocytogenes* detection using PALCAM plating medium

After selecting the samples, 20 g is added to bags with 180 ml of BPW (Buffered Peptone Water). Incubation lasts one hour at a temperature of 20 °C. After one hour of incubation at room temperature, 0.1 ml is taken from the prepared culture and inoculated with ALOA and PALCAM with a stick. Then, it is incubated again for 24 hours at 37 °C. After incubation for 24 hours, it is necessary to additionally incubate ALOA and PALCAM for another 24 hours.

ALOA agar positive for *L. monocytogenes* shows green-blue colonies surrounded by a cloudy areola, while PALCAM shows typical grey-green colonies with a black areola.

ChromoCult *Listeria* agar is prepared by separating 35 g of the medium and adding it to 500 ml of distilled water. The ingredients should be well mixed and dissolved by heating with frequent shaking. Then it is necessary to boil the agar for one minute until it dissolves completely. The next step is autoclaving for 15 minutes at 121 °C, and, then cooling to 70 °C and aseptically adding of one bottle of *Listeria* Chromogenic Selective Supplement, previously reconstituted in 6 ml of warm and sterile distilled water. After that, the contents are gently homogenized and dispensed into Petri dishes.

The procedure is similar for PALCAM *Listeria-Selective* agar. Primarily, it is necessary to separate 34.4 g of media, which are then added to 500 ml of distilled water. Mixing well and heating for dissolution with frequent shaking are required. The content should be boiled for one minute until completely dissolved. After that, the solutions are placed in an autoclave for sterilization for 15 minutes at 121 °C. Sterilization is followed by

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cooling at 45–50 °C and aseptically adding one bottle of PALCAM *Listeria* Selective Supplement, previously reconstituted in 5 ml of sterile distilled water. The content should be gently homogenized and dispensed into Petri dishes.

Additional microbiological testing was performed at the Veterinary Institute of Herzegovina–Neretva Canton in Mostar. Analyses to confirm the presence of *L. monocytogenes* were as follows:

- Gram staining is performed after isolating colonies, whereby *Listeria* spp. and *Listeria monocytogenes* show as Gram-positive thin, short rods. This staining is mandatory for confirmation of *Listeria* spp. and optional for *Listeria monocytogenes*.
- A haemolysis test is obligatory for *Listeria monocytogenes* bacteria. After incubation at 37 °C for 24 ± 2 hours, test strains and control cultures are examined. *Listeria monocytogenes* shows a narrow and bright zone (*β*-haemolysis), while *Listeria innocua* does not show a bright zone around the sting. *Listeria ivanovii* typically shows a wide and distinctly delimited zone of *β*-haemolysis. Finally, the Petri dishes are examined under white light to compare the tested with control cultures.
- The CAMP test is optional to confirm the presence of *Listeria monocytogenes* bacteria. It begins by streaking each of the cultures of *Staphylococcus aureus* and *Rhodococcus equi* as a single line across Petri dishes of sheep blood agar so that the two cultures are placed parallel and diametrically opposite.

After that, the control cultures of *Listeria monocytogenes*, *Listeria innocua*, and *Listeria ivanovii* are simultaneously sown in furrows. Petri dishes are incubated at 37 °C for 18 to 24 hours. A positive reaction is shown as an increased zone of *β*-haemolysis on the cross-section of the test strain with each of the cultures of *Staphylococcus aureus* and *Rhodococcus equi*. A positive reaction with *Rhodococcus equi* is mostly shown as a wide zone of haemolysis (5 to 10 mm) in the shape of an ″arrowhead″. A positive reaction with *Staphylococcus aureus* appears as a small zone of increased haemolysis extending only about 2 mm from the test strain and within a weak haemolytic zone due to the growth of the *Staphylococcus aureus* culture. Large zones of haemolysis do not appear in the area of *Staphylococcus aureus* and *Listeria monocytogenes*.

- The catalase reaction is a mandatory test for the confirmation of *Listeria* spp. and optional for the confirmation of *Listeria monocytogenes*. It is done by taking one isolated colony on TSYEA (tryptone soy yeast extract agar) and suspending it in a drop of hydrogen peroxide solution H_2O_2 on a microscope slide. Immediate bubbling indicates a positive reaction.
- A test using carbohydrates is mandatory to confirm *Listeria monocytogenes*. The test is performed by inoculating each carbohydrate consumption broth with a culture on TSYEA (tryptone soy yeast extract agar) using an inoculating loop.

An online survey was conducted in the area of Mostar on a sample of 419 respondents aged 16 to 60+. It was completed by filling out a questionnaire consisting of 11 questions.

The survey aimed to find out how familiar the wider population is with the pathogen *Listeria monocytogenes*. The aim was to test the knowledge about the disease listeriosis that this pathogen causes, whether good hygiene practices are used, and are the basis of food safety followed to obtain a product safe for consumption.

3. Results and Discussion

After 24 hours of incubation, some samples indicated the appearance of colonies that looked like those of *Listeria* bacteria but were atypical for *Listeria monocytogenes*. Also, some samples revealed the presence of atypical unknown colonies.

Table 1. Representation of the results of washed vegetables from the laboratory of the Federal Agro-Mediterranean Institute on ALOA and PALCAM supplement plating media

Ordinal number	**Sample name**	**PALCAM**		**ALOA**	
		Dilution used		Dilution used	
		10^{-1}	10^{-2}	10^{-1}	10^{-2}
1st	P1T	Ø	Ø	Ø	Ø
2nd	P1Te	Ø	Ø	Ø	Ø
3rd	P1MFM	Ø	Ø	Ø	Ø
4th	P1B	Ø	Ø	Ø	Ø
5th	PA1T	Ø	Ø	Ø	Ø

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6th	PA1Te	Ø	Ø	Ø	Ø
7th	PA1MFM	Ø	Ø	Ø	Ø
8th	PA1B	Ø	Ø	Ø	Ø

Table 2. Representation of the results of unwashed vegetables from the laboratory of the Federal Agro-Mediterranean Institute on ALOA and PALCAM supplement plating media

Ordinal number	**Sample name**	**PALCAM**		**ALOA**	
		Dilution used		Dilution used	
		10^{-1}	10^{-2}	10^{-1}	10^{-2}
1st	P2T	78	An increase in colonies was observed, but they are atypical for *L. monocytogenes*	An increase in colonies was observed, but they are atypical for *L. monocytogenes*	An increase in colonies was observed, but they are atypical for *L. monocytogenes*
2nd	P2Te	Ø	Ø	Ø	Ø
3rd	P2MFM	Ø	Ø	Ø	Ø
4th	P2B	Ø	Ø	Ø	Ø
5th	PA2T	8	An increase in colonies was observed, but they are atypical for *L. monocytogenes*	An increase in colonies was observed, but they are atypical for *L. monocytogenes*	An increase in colonies was observed, but they are atypical for *L. monocytogenes*
6th	PA2Te	Ø	Ø	Ø	Ø
7th	PA2MFM	Ø	Ø	An increase in colonies was observed, but they are atypical for *L. monocytogenes*	An increase in colonies was observed, but they are atypical for *L. monocytogenes*
8th	PA2B	1 colony over one-half of the plate area	1 colony over one-half of the plate area	An increase in colonies was observed, *L.* spp	An increase in colonies was observed, but they are atypical for *L.* spp

To confirm whether it is *Listeria monocytogenes*, all samples with atypical colonies continued to be tested additionally in the laboratory of the Veterinary Institute of Herzegovina–Neretva Canton in Mostar.
Gram staining did not give positive results. All samples were placed in xylose and rhamnose sugars used to compare *Listeria* strains. The catalase reaction was positive.
After completing haemolysis tests, CAMP test, and carbohydrate utilization test, the samples showed negative results. Haemolysis was not present. The CAMP test did not react typically for the bacterium *Listeria monocytogenes*.
Regarding the carbohydrate test, this test gave an atypical reaction for the bacterium *Listeria monocytogenes*, and it reacted positively with the xylose sugar, while the reaction remained negative with rhamnose.
All confirmations were made by consistently comparing the results with the positive sample. The certified reference material used during the analysis is *Listeria monocytogenes* WDCM 00021.
A study that began in December 2006, in Malaysia, says that *Listeria* was found in raw tomatoes. Samples were collected at street markets. (Jamali, H. et al., 2013)
Berrada et al., in 2006, analysed 77 pre-made salads served in restaurants in Valencia, Spain. The aim was to implement a method for quantifying *L. monocytogenes* in salads using real-time PCR quantification. The results were compared with classical detection methods. Both methods showed the presence of *L. monocytogenes* in 3.9 percent of the samples.

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The so-called ″Summer salad″, made with potatoes, onions, tomatoes, olive oil, and salt, showed the presence of pathogens in 1 out of 25 samples. Meanwhile, the so-called ″Valencian salad″, which consists of tomatoes, onions, lettuce, olives, tuna, and boiled eggs, showed the presence of the pathogen *L. monocytogenes* in 2 out of 28 samples.

Between 2002 and 2010, a quantitative analysis of the presence of *L. monocytogenes* in cafeterias and restaurants was conducted in Valencia, Spain. In total, 2 262 samples of cold meat products, eggs, fish products, mayonnaise, and salads were tested. Microbiological analysis showed the presence of *L. monocytogenes* in 0.1 percent of the samples. Despite the zero percentage of positive results, the authors concluded that constant monitoring during food preparation, production, and serving is necessary to keep the rate of positive samples low (Domenech, E. et al., 2011).

In 2008, Jalali and Abedi reported a low prevalence of. monocytogenes (1.2%) in vegetables in Isfahan province, Iran (Jalali and Abedi, 2008)

The primary causes of the occurrence of *Listeria monocytogenes* bacteria are the poor microbiological quality of raw food, contamination, cross-contamination, inadequate cleaning, poor hygiene practices, unsafe storage temperature, inadequate preparation process, contamination during transportation, and lack of staff training in food hygiene.

Survey Results

Results of 419 respondents:

- More than half of the respondents consume vegetables (specifically tomatoes and peppers) one to three times a week (52%)
- The respondents mostly buy tomatoes and peppers in stores (36%)
- The respondents mostly consume fresh vegetables (62%)
- The vast majority of respondents always washed vegetables thoroughly before consumption (95%)
- The vast majority of respondents had not heard of the bacterium *Listeria monocytogenes* (62%)
- According to the previous question, the majority of respondents did not know what listeriosis is (69%)
- The answers to the final question indicate that the majority of respondents did not know how listeriosis manifests itself (73,5%)

4. Conclusion

The bacterium *Listeria monocytogenes* imposes a great danger in food precisely because of its pathogenicity and the ability to survive in various poor conditions. For this reason, it is necessary to maintain hygiene regularly and correctly, both in kitchens and in food processing plants, and to carry out the adequate processing procedures of raw materials to avoid contamination of products intended for the market and, thus, the occurrence of listeriosis, which can be deadly.

It is obvious that good hygiene practice, as one of the main steps in the preparation and production of food, prevents the occurrence of *Listeria monocytogenes* because all the samples that had formed atypical colonies were unwashed samples. Also, regular monitoring is necessary to provide safe and healthy food on the market.

The results of the survey show that the population in the area of the City of Mostar is predominantly not familiar with the bacterium *Listeria monocytogenes* and its occurrence in food. It would be necessary to hold lectures in educational institutions, as well as educational seminars on the dangers that are present when consuming food.

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A Brief Author Biography

1st Author Name – M.Sc. Mirza Kovačić, University „Džemal Bijedić“, Agromediterraneane Faculty, 88104 Mostar. He completed two summer schools that are part of the cooperation achieved by the Czech University of Life Sciences Prague, the University of "Džemal Bijedić" in Mostar and the University of Mostar. As part of his education, he showed the greatest interest in food toxicology. Guided by his interests, he chose the topic "Listeria monocytogenes bacteria: occurrence in vegetables and health risks" for his graduation thesis.

2nd Author Name – PhD. Aida Šukalić, Associate professor for the narrower scientific field of Food Safety at University „Džemal Bijedić“, Agromediterraneane Faculty, 88104 Mostar. During her work, she showed interest and inclination towards scientific and research work. She published 35 scientific and research papers as an author and co-author, and participated in symposia and projects related to the profession. She is the author of the book: "Mycotoxins - biological food contaminants" (2020).

