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Study of the cultural properties of *Listeria monocytogenes / Listeria spp* under different storage temperature regimes

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Abstract. *Microbial contamination of food products remains a major public health threat, as consumption of contaminated foods can cause infectious diseases. Among pathogenic bacteria,* Listeria monocytogenes *occupies a leading position. The significance of listeriosis, caused* by *L. monocytogenes, is determined by its epizootic, economic, and ecological impact. Pathogenic species of* Listeria *have become permanent contaminants of food raw materials and food products. Ensuring compliance with microbiological safety requirements remains a critical challenge for food producers, particularly concerning L. monocytogenes contamination. Listeria monocytogenes is a pathogen capable of surviving storage conditions at low temperatures (−25 °C) and high temperatures (+45 °C). Microorganisms remain viable during prolonged storage in food products at processing plants. Understanding the resistance mechanisms of L. monocytogenes provides essential insight for improving food processing and preservation technologies. The study provides a comprehensive overview of the properties of pathogenic Listeria (at the species level) under different temperature conditions and during long-term storage, which may lead researchers to further implement innovative technologies for the elimination of pathogenic* Listeria monocytogenes *in food production. Scientists are currently developing new methods for eliminating bacterial contamination in food products and in food industry environments.*
Key words: *Listeria monocytogenes*, isolate, microorganism, strain, quality, CAMP test, hemolytic test, food samples, CFU (colony-forming units)

Listeria monocytogenes are widely distributed facultative anaerobic bacteria belonging to the family *Listeriaceae* and the genus *Listeria*. *L. monocytogenes* causes the infectious disease listeriosis and is the only species in the genus considered pathogenic for both humans and animals. These microorganisms are found commonly in soil, water, feed, and various food products. The list of animal reservoirs is broad and includes wild and domestic animals, as well as birds. Infected animals excrete *Listeria* into the environment through urine, feces, nasal secretions, and milk. Food products such as milk, meat, fish, vegetables, and fruits provide an excellent nutrient medium for the growth and reproduction of these bacteria (Bahramia, 2020; Shostakovich-Koretska, 2021).

Listeriosis is considered an emerging zoonotic disease due to the increasing prevalence of *Listeria* in nature and its ability to parasitize intracellularly, which contributes to chronic or asymptomatic infections (asymptomatic carriage of *Listeria*) (Sorokina, 2016).

Morbidity, costs of preventive and therapeutic measures, and reduced animal productivity lead to significant economic losses. Therefore, there is a need to study ways to create highly effective diagnostic and preventive measures against listeriosis (Chen, 2017).

Foodborne listeriosis represents a serious issue that often results in the withdrawal of contaminated batches of products, trade restrictions, and even production shutdowns. To ensure food safety, regular laboratory monitoring is required at all stages of the production process, from raw materials and equipment to production facilities and personnel (Iacumin, 2017). Listeriosis is not only a veterinary concern but also a significant public health problem (Kramarenko, 2013).

According to the results of *Listeria monocytogenes* monitoring conducted by the Bacteriology Research Department of the State Research Institute for Laboratory Diagnostics and Veterinary and Sanitary Expertise (Kyiv, Ukraine) during 2020–2024, the following findings were obtained:

- 143,416 samples of domestically produced products were tested, and 258 isolates of *L. monocytogenes* were detected.
- 64,545 samples of export products were tested, yielding 9 isolates of *L. monocytogenes*.
- 36,637 samples of imported products were tested, yielding 14 isolates of *L. monocytogenes*.
- 8,950 samples under state control were tested, yielding 23 isolates of *L. monocytogenes*.

Table 1

Microbiological testing of food products in Ukraine, 2020–2024

Years	Domestic production		Export products		Imported products		State-controlled products	
	Number of tests	*Listeria monocytegenes* isolation cases	Number of tests	*Listeria monocytegenes* isolation cases	Number of tests	*Listeria monocytegenes* isolation cases	Number of tests	*Listeria monocytegenes* isolation cases
2020	42393	76	11750	2	14926	2	4390	16
2021	34539	51	14289	6	15826	0	2734	5
2022	17445	14	9132	1	4751	0	167	0
2023	22489	73	12502	0	240	9	628	0
2024	26550	44	16872	0	894	3	1031	2
Total	143416	258	64545	9	36637	14	8950	23

The testing of food products and detection of *L. monocytogenes* microorganisms remains a pressing issue, but counting its presence in a product is no less important. The degree of product contamination can vary, and therefore the safety of human consumption depends on it (Buchanan, 2020).

Food safety is governed by EU Regulation No. 2073/2005, which specifies the limits for the generally acceptable level of microorganisms, namely the number of colony-forming units—100 CFU per 1 g (1 ml) of product (CFU/g, cm³). The presence of *L. monocytogenes* in 25 g (25 cm³) of food products ready for consumption is not permitted. The exception is products for infants (Gray, 2019).

The safety of a food product is assessed based on the standardized weight of the product, in which the presence of the pathogenic microorganism *L. monocytogenes* is not permitted, or the number of colony-forming units must not exceed 100 CFU per 1 g (1 ml) of product (CFU/g, cm³).

The presence of microorganisms in food products, including *Listeria monocytogenes*, causes food spoilage. Food spoilage is a metabolic process that makes food undesirable or unacceptable for human consumption. Food products are a nutrient medium for microorganisms in general, including *L. monocytogenes*. Microorganisms cause fermentation and decomposition of products and release substances that are unpleasant to the taste and often toxic (Feng, 2020). Microorganisms of the genus *Listeria* are most commonly found in foods that do not undergo heat treatment. In recent years, there has been a significant increase in the market for ready-to-eat foods, such as ready-to-eat (RTE), ready-to-heat (RTH), and ready-to-cook (RTC) meat products (Oshchypok, 2020; Kanmani, 2014).

The rate of microbial growth is influenced by temperature, acidity (pH), and the availability of nutrients. The most favorable temperature for *Listeria* growth is between +2 °C and +45 °C (Kawacka, 2020).

The aim of the study. It was to conduct microbiological studies of food products with indication of microorganisms of the genus *Listeria*, determine the cultural and biochemical properties of *Listeria spp.* isolates with subsequent identification to the species level, and study the resistance of *L. monocytogenes* isolates to changes in environmental conditions.

Materials and methods. Experimental studies were conducted at the laboratory for microbiological research of food products and feed of the scientific and research bacteriological department of the State Research Institute for Laboratory Diagnostics and Veterinary and Sanitary Expertise (SSRILDVSE), which is accredited by the National Accreditation Agency of Ukraine for competence in accordance with the requirements of DSTU ISO/IEC 17025-21.

The material for the study was food products, namely domestically produced, exported, imported, and state-controlled products. For further species identification of the isolates, the morphological and biochemical properties of the microorganisms were studied.

The study of the resistance of isolates to changes in environmental conditions was carried out only for *L. monocytogenes* microorganisms.

A test batch of contaminated food samples was placed in controlled storage in a refrigerated environment at a temperature of +8.0 °C ± 1.0 °C for 7 days and, at the same time, was placed in controlled storage in a freezer compartment at a temperature of −25.0 °C ± 1.0 °C for the same period. After 7 days of storage, the food products from the two parallel storage batches were examined. The microbiological indicators of the experimental samples were evaluated for the presence of *L. monocytogenes*.

Microbiological studies of experimental samples, examination of properties, analysis, and interpretation of the results for *L. monocytogenes* microorganisms were carried out in accordance with current international documentation (ISO 11290, 2017). Buffered peptone water (HiMedia M614) was used as a solvent to prepare the initial suspension. From each batch of infected material, 10 cm^3 of test sample was taken and placed in a sterile BagFilter bag, and 90 cm^3 of solvent was added. After homogenizing the suspension on a BagMixer homogenizer, a series of serial dilutions (up to $1:10^4$ inclusive) were prepared (ISO 7218, 2024).

Using a sterile pipette (0.1–0.3 cm^3), the initial suspension was applied to each of six cups containing *Listeria* mono agar (ALOA) (HiMedia M2009). The procedure was repeated using tenfold dilutions. The inoculum was carefully spread over the surface of the agar plate with a spatula. The cultures were incubated in a thermostat at 37.0 ± 1.0 °C for 24–48 hours. At the end of the incubation period, all similar *L. monocytogenes* colonies on each plate were counted. To confirm the purity of the culture, five similar typical colonies were selected from each dish and transferred to dishes with agar medium (HiMedia M001) and cultivated at a temperature of 37.0 ± 1.0 °C for 16–24 hours. The morphological and biochemical properties of *Listeria monocytogenes* microorganisms were studied using a microscopic method (Gram staining), a hemolytic test, a CAMP test, a catalase test, and a carbohydrate fermentation test (ISO 11290, 2017).

Results. In the first quarter of 2024, 15 *Listeria spp.* isolates were identified in food products. As a result of the research, the isolates had typical colonies characteristic of the *Listeria* genus. Morphological and biochemical studies were conducted for further identification of microorganisms.

During microscopy, Gram-positive rod-shaped bacteria of regular shape were observed in the microscope field of view, located singly and in clusters.

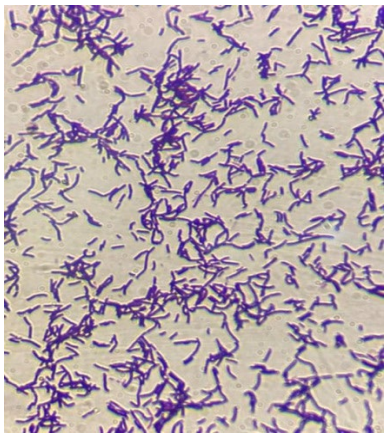

Fig. 1. Gram stained *Listeria monocytogenes* from one day agar culture

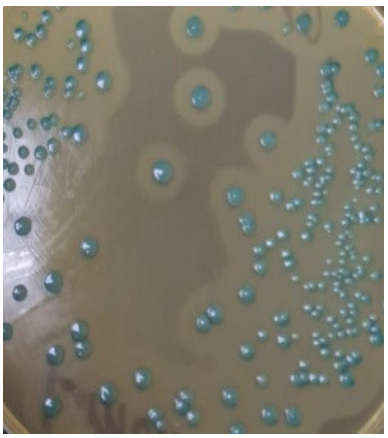

Fig. 2. Growth of *Listeria monocytogenes* on medium *L. mono* (ALOA)

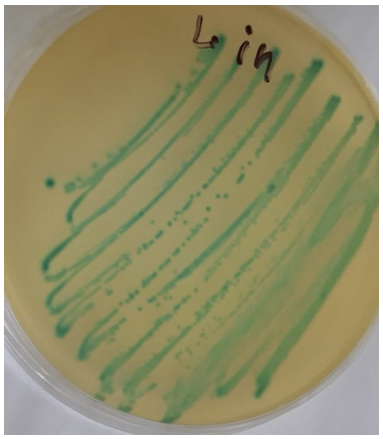

Fig. 3. Growth of *Listeria innocua* on medium *L. mono* (ALOA)

For species identification of *Listeria spp.* isolates, the CAMP test was performed in accordance with ISO 11290-2:2017. *Staphylococcus aureus* ATCC 25923, *Rhodococcus egvi* ATCC 6939, and *L. monocytogenes* ATCC 33090 test cultures were used for test control. Daily cultures of *Staphylococcus aureus* and *Rhodococcus egvi* strains were seeded on blood agar (Columbia agar + 5% sheep blood) so that the cultures were diametrically opposite. Immediately, experimental daily agar cultures of *Listeria spp.* microorganisms were sown between the vertical lines of *Staphylococcus aureus* ATCC 25923 and *Rhodococcus egvi* ATCC 6939 strains (the distance from the vertical line of strains to the experimental culture was at least 5 mm). The dishes were cultured at a temperature of 37.0 ± 1.0 °C for 12–18 hours. After 24 hours, a zone of hemolysis was present on the dish near the inoculation of the test culture *Staphylococcus aureus* (positive reaction) and *Rhodococcus egvi* (negative reaction). These properties are characteristic of *L. monocytogenes* microorganisms (Fig. 4). In addition, there was no zone of hemolysis on the Petri dish near the test culture *Rhodococcus egvi* and the test culture *Staphylococcus aureus*. These properties are characteristic of microorganisms of the species *L. innocua* (Fig. 7).

A catalase test for *Listeria spp.* microorganisms was performed, which showed a positive result.

To study hemolytic properties, test samples were cultured on blood agar (Columbia agar + 5% sheep blood). A test culture of *L. monocytogenes* ATCC 33090 was sown for control purposes. The cups were cultured in a thermostat at a temperature of 37.0 ± 1.0 °C for 24 hours. The *Listeria spp.* microorganisms in the test samples had a light zone of β-hemolysis around the colonies, which is characteristic of the *L. monocytogenes* species (Fig. 5). In some *Listeria spp.* microorganisms, the hemolysis zone was absent, which may be attributed to the *Listeria innocua* species.

When studying carbohydrate fermentation, daily agarized colonies of *Listeria spp.* from test samples were cultured in tubes with broth medium (Phenol Red Broth Base) (HiMedia M054), and xylose and rhamnose discs were immersed in the broth and cultured in a thermostat at a temperature of 37.0 ± 1.0 °C for 24–48 hours.

After 2 days, changes were observed in the tubes with broth medium, namely a change in color to yellow (acid formation). With the growth of *Listeria spp.* microorganisms in the rhamnose broth, the color changed to yellow, while in the xylose broth the color remained unchanged (Fig. 6). These properties are characteristic of microorganisms of the species *L. monocytogenes*. In test tubes with xylose and rhamnose broth, when several *Listeria spp.* microorganisms were seeded, no changes were observed after cultivation, which is characteristic of microorganisms of the species *L. innocua*.

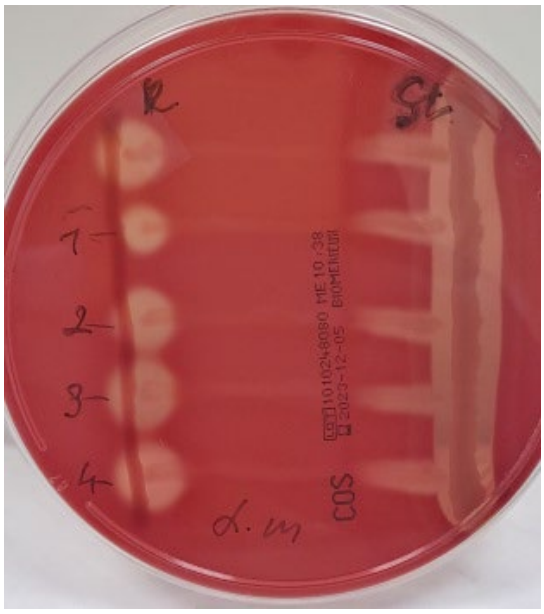

Fig. 4. CAMP test of experimental samples *Listeria monocytogenes*

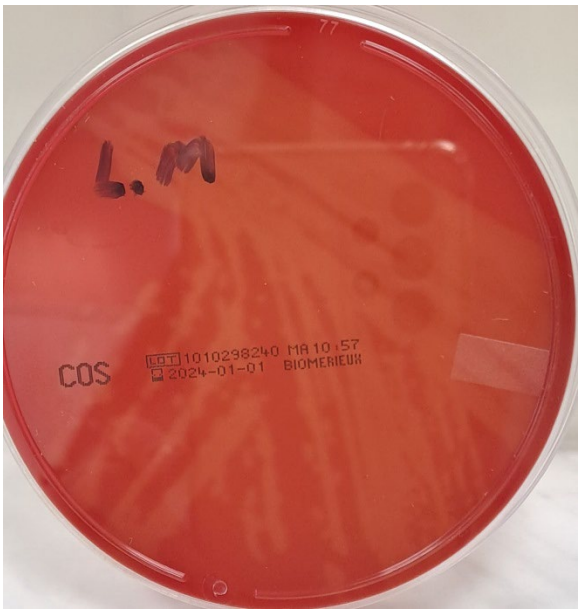

Fig. 5. Hemolytic test of tested samples

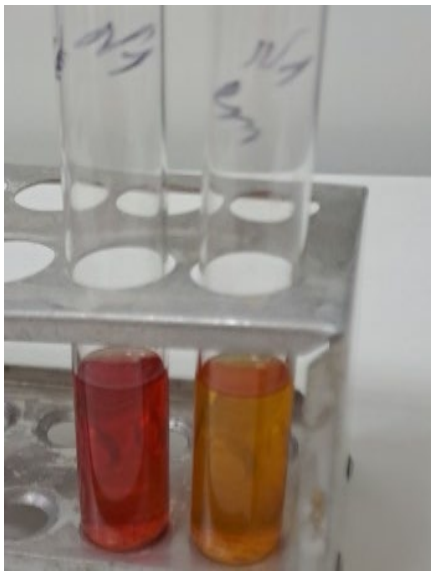

Fig. 6. Growth of *Listeria monocytogenes* in broth medium with Xylose and Rhamnose discs

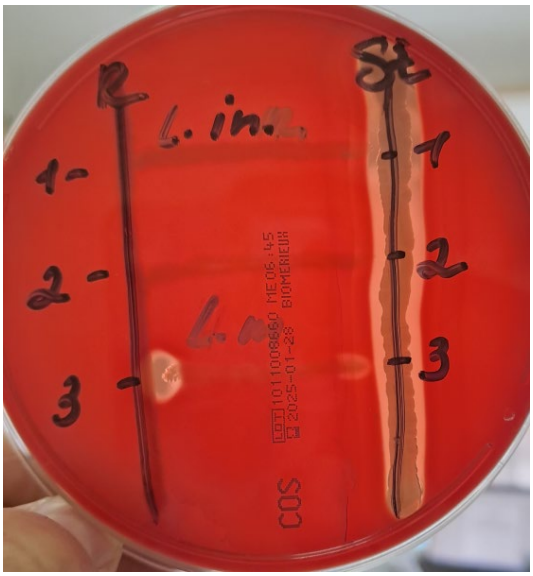

Fig. 7. CAMP test of experimental samples of *Listeria monocytogenes and Listeria innocua*

Discussion. As a result of the research, 15 isolates of the *Listeria* family were identified in food products, 10 of which belong to the species *L. monocytogenes* (Fig. 2) and 5 to the species *L. innocua* (Fig. 3).

Since *L. monocytogenes* is the main pathogen in food products for humans, scientists are particularly interested in its resistance to various environmental conditions. Therefore, we conducted a study to investigate the resistance of *L. monocytogenes* isolates to different storage temperature regimes.

To study the resistance of *L. monocytogenes* isolates to changes in environmental conditions, microorganisms were cultured on Petri dishes with *Listeria* mono agar (ALOA). On the L. mono medium (tenfold dilutions), the growth of round typical colonies with a green color and a halo in the center was observed *L. monocytogenes* (Fig. 2).

Colony counting and interpretation of *Listeria monocytogenes* results in 1 ml (CFU/cm³) were performed using the following formula:

$$N=\frac{\sum a}{V\cdot(n_1+0{,}1\cdot n_2)\cdot d},$$

where ∑a is the sum of typical *L. monocytogenes* colonies counted after confirmation on all plates in the last two consecutive dilutions;
V is the volume of the sown sample in each cup in cm³;
n_1 is the number of cups that grew colonies in the first dilution;
n_2 is the number of cups that grew colonies in the second dilution;
d is the dilution coefficient.

The results obtained regarding the number of microorganisms were expressed as colony-forming units (CFU/cm³).

The study of samples showed uniform growth of *Listeria monocytogenes* microorganisms in 1 ml of product (CFU/cm³), where the average number of microorganisms was 6.3 × 10³ (Fig. 9). When examining samples after 7 days of storage in the refrigerator and freezer compartments, visible changes in the number of *Listeria monocytogenes* colonies in 1 ml of product (CFU/cm³) were observed (Fig. 10).

A comparison was made of ten selected values relating to two data sets, namely the results of sowing the number of *Listeria monocytogenes* colonies in test samples stored for 7 days in the refrigerator compartment at a temperature of +8.0 °C ± 1.0 °C and in the freezer compartment at a temperature of −25.0 °C ± 1.0 °C, to study how much the average values differ significantly from each other at different storage temperature regimes using Student's *t*-criterion (Petruk, 2022).

Using Student's criterion, we determined how visible the differences between the two values were. This parametric method is used to test hypotheses about the reliability of differences between mean values when analyzing quantitative data in general populations with normal distribution and equal variance (Malaychuk, 2022).

The algorithm for calculating Student's *t*-criterion (*t*) was performed using the following formulas:

$$\sigma=\sqrt{\sigma x^2+\sigma y^2}=\sqrt{\frac{\sum(x-\overline{x})^2+\sum(y-\overline{y})^2}{n_1+n_2-2}}\times\sqrt{\frac{n_1+n_2}{n_1\times n_2}},$$

where **x** is the number of *Listeria monocytogenes* colonies in test samples examined after 7 days of cold storage at a temperature of +8.0 °C ± 1.0 °C;
y is the number of *Listeria monocytogenes* colonies in test samples examined after 7 days of storage in the freezer compartment at a temperature of −25.0 °C ± 1.0 °C;
n is the number of research samples;
σ is the average deviation of values between the number of *Listeria monocytogenes* colonies in the test samples examined after seven days of storage in the refrigerator compartment at a temperature of +8.0 °C ± 1.0 °C and in the freezer compartment at a temperature of −25.0 °C ± 1.0 °C.

Average values of *L. monocytogenes* colony count:
$\bar{x}$ = 19,800
$\bar{y}$ = 501

Calculation of the difference in absolute value between average values:
$|\bar{x}-\bar{y}|$ = 19,299

Calculation of the variance of mean deviations:
σ = 1,533.6.

Then: *t* = 12.5.

Number of degrees of freedom: df = 18.

According to the table of critical values of the Student's *t*-distribution (*t*-criterion) for a given number of degrees of freedom, we find:

$$T_{cr} = \begin{cases} 2,10 \text{ for } \leq 0,05 \\ 2,88 \text{ for } \leq 0,01 \\ 3,92 \text{ for } \leq 0,001 \end{cases}$$

The reliability of differences exists.

Thus, the similarity of the results for the number of *Listeria monocytogenes* colonies grown according to these indicators differs, since at the level of significance there are differences between the number of *Listeria monocytogenes* colonies grown in the test samples which were examined after seven days of storage in the refrigerator compartment at a temperature of +8,0 ºC ± 1,0 ºC and in the freezer compartment at a temperature of - 25,0 º C ± 1,0 º C (Fig. 8).

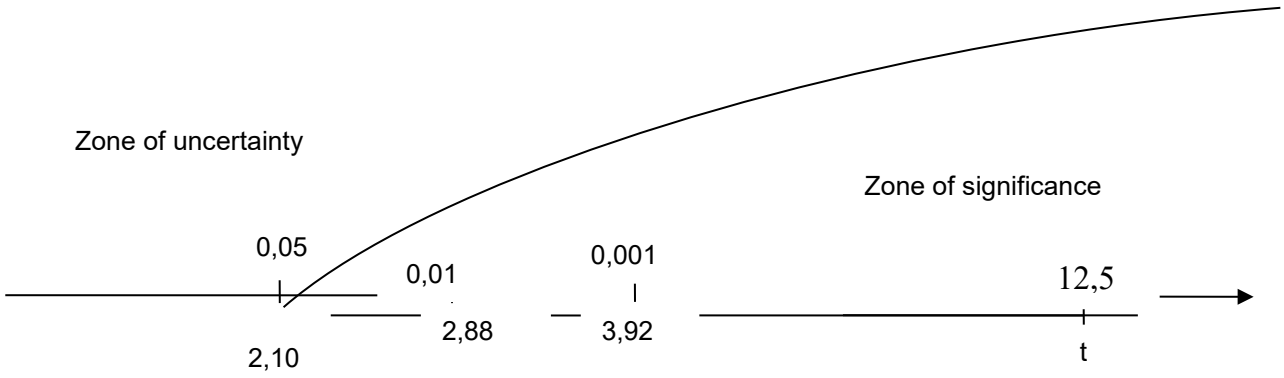

Fig. 8. Significance axis of the results of the study of *L. monocytogenes* microorganisms in test samples under different temperature conditions and storage periods

Table 2

Characteristics of *L. monocytogenes* microorganisms in 1 ml (CFU/cm3) of test samples when stored at different temperatures

Experimental samples	Initial results of sowing experimental samples (ES)	Results of sowing DZ after 7 days of cold storage at a temperature +8,0ºC ± 1,0ºC	Results of sowing DZ after 7 days of freezer storage at a temperature - 25,0ºC ± 1,0ºC
	number of colonies		
	$1:10^3$	$1:10^4$	$1:10^2$
№1	6000 ± 0,37	26000 ± 0,40	580 ± 0,17
№2	6900 ± 0,56	14000 ± 0,37	620 ± 0,34
№3	7100 ± 0,37	23000 ± 0,56	460 ± 0,21
№4	5800 ± 0,43	17000 ± 0,48	410 ± 0,31
№5	6700 ± 0,31	12000 ± 0,49	600 ± 0,33
№6	5000 ± 0,52	21000 ± 0,48	570 ± 0,43
№7	8200 ± 0,33	25000 ± 0,60	400 ± 0,42
№8	4200 ± 0,26	16000 ± 0,26	450 ± 0,49
№9	6500 ± 0,37	20000 ± 0,37	580 ± 0,54
№10	5700 ± 0,40	24000 ± 0,40	340 ± 0,52

$p < 0,05$

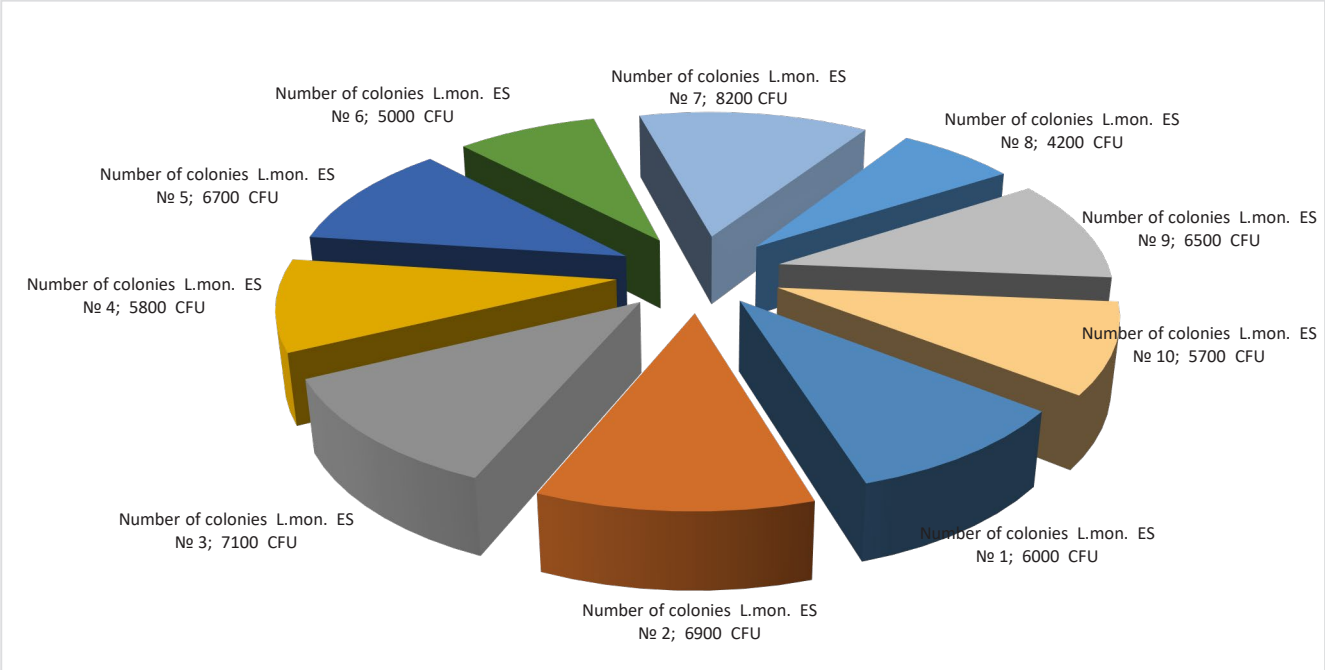

Fig. 9. Results of the growth of *L. monocytogenes* microorganism colonies in primary test samples

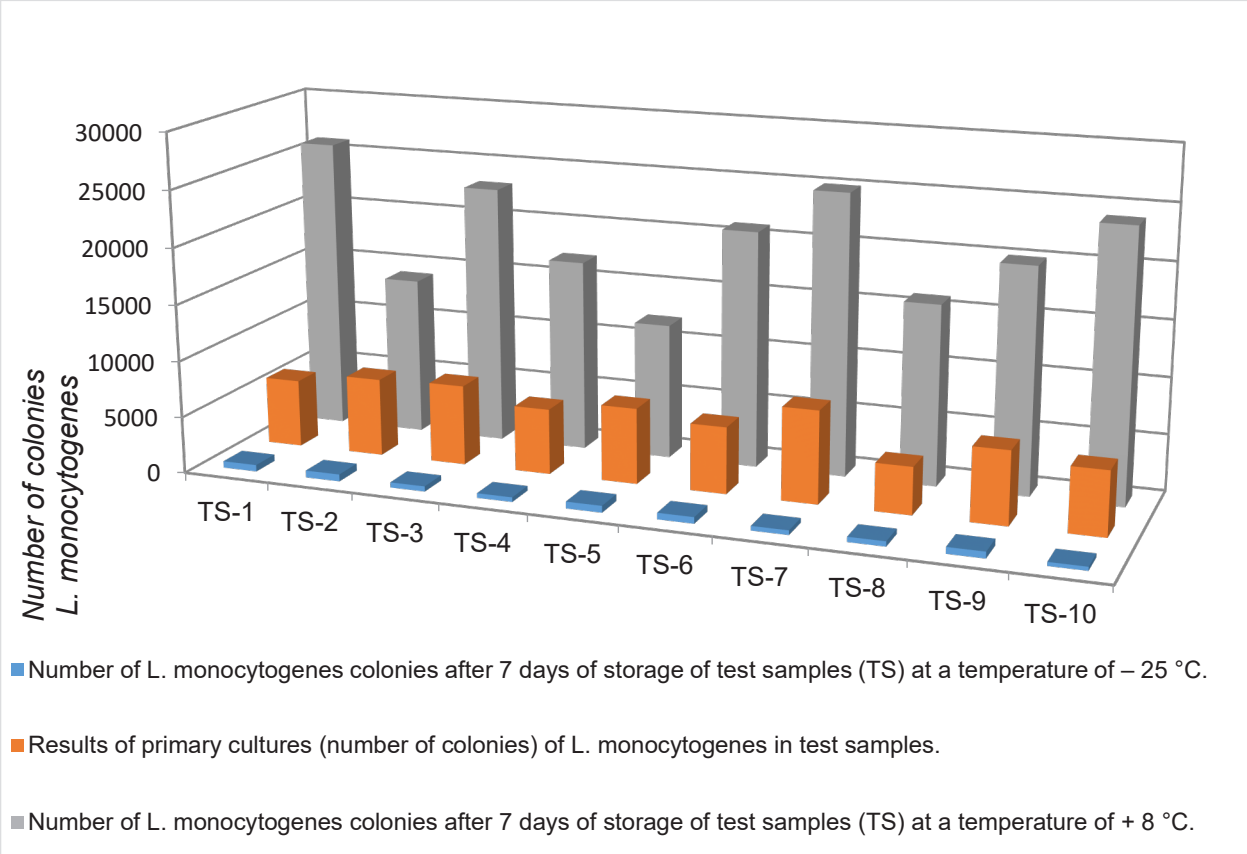

Fig. 10. Results of *L. monocytogenes* microorganism cultures at different stages of the study under different temperature conditions

Conclusions. As a result of microbiological testing of food samples, 15 microorganisms of the genus *Listeria* were detected.

The cultural and biochemical properties of 15 isolates of *Listeria* spp. were studied.

The obtained isolates of *Listeria* spp. were identified to species level. As a result of performing the CAMP test on a Petri dish with blood agar, 10 isolates showed a zone of β-hemolysis near the test culture of *Staphylococcus aureus* (positive reaction); in 5 isolates, the hemolysis zone was absent. The results of the study of 15 isolates in terms of carbohydrate utilization confirm that isolates with pronounced β-hemolytic properties actively oxidized rhamnose. As a result, they were identified as *L. monocytogenes*.

During the study period, a trend was observed in contaminated samples that reflects the growth activity of *L. monocytogenes* under various storage conditions:

- The primary (directly obtained) average value is 6.3×10^3 CFU/g ± 0.39 (Colony-forming unit).
- Refrigerated storage at +8.0 ºC ± 1.0 ºC, the average value is 2.0×10^4 CFU/g ± 0.44.
- Freezer storage at -25 ºC ± 1.0 ºC, the average value is 5.0×10^2 CFU/g ± 0.38.

Therefore, freezing storage of products at -25.0 ºC ± 1.0 ºC for 7 days reduced *L. monocytogenes* contamination in 1 cm^3 (1 g) of product by approximately 91.9%, and storage of food products in a refrigerator at +8.0 ºC ± 1.0 ºC for 7 days increased the contaminant content by approximately 222.6% compared to the initial study.

The results obtained demonstrate that the growth of *L. monocytogenes* colonies in 1 g (1 cm^3) or 25 g (25 cm^3) of product (CFU/g, CFU/cm^3), in accordance with EU Regulation No. 2073/2005, depends on the temperature regime and duration of storage of the contaminated product.

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Вивчення культуральних властивостей *Listeria monocytogenes* / *Listeria* spp за різних температурних режимів зберігання

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Резюме. *Мікробне забруднення харчових продуктів залишається серйозною загрозою для здоров'я населення, оскільки споживання забруднених продуктів може спричинити інфекційні захворювання. Серед патогенних бактерій Listeria monocytogenes займає провідне місце. Значення лістеріозу, спричиненого L. monocytogenes, зумовлене його епізоотичним, економічним та екологічним впливом. Патогенні види Listeria стали постійними забруднювачами харчової сировини та харчових продуктів. Забезпечення дотримання вимог мікробіологічної безпеки залишається критичним завданням для виробників харчових продуктів, особливо щодо забруднення L. monocytogenes. Listeria monocytogenes – це патоген, здатний виживати в умовах зберігання за низьких температур (-25°C) та високих температур (+45°C). Мікроорганізми залишаються життєздатними під час тривалого зберігання в харчових продуктах на переробних підприємствах. Розуміння механізмів резистентності L. monocytogenes дає важливе розуміння для вдосконалення технологій переробки та консервування харчових продуктів. Дослідження надає всебічний огляд властивостей патогенних Listeria (на видовому рівні) за різних температурних умов та під час тривалого зберігання, що може спонукати дослідників до подальшого впровадження інноваційних технологій для ліквідації патогенних Listeria monocytogenes у виробництві харчових продуктів. Вчені зараз розробляють нові методи усунення бактеріального забруднення харчових продуктів та в умовах харчової промисловості.*

Ключові слова: *Listeria monocytogenes*, ізолят, мікроорганізм, штам, якість, CAMP-тест, гемолітичний тест, зразки харчових продуктів, КУО (колонієутворюючі одиниці)

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