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PREVALENCE OF *LISTERIA MONOCYTOGENES* IN READY-TO-EAT FOODS IN BULGARIA

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Abstract

Listeria monocytogenes is a widespread foodborne pathogen, which is able to grow and survive even at refrigeration temperatures, in vacuum-packed food and in modified atmosphere. It is also often found as an environmental contaminant in food processing plants. Important sources of *L. monocytogenes* are different ready-to-eat (RTE) products with a long shelf life. RTE are also known as products which support the growth of *L. monocytogenes*. The microbiological criteria for *L. monocytogenes* in foodstuffs are specified in Commission Regulation (EC) No 2073/2005. For RTE foods which are able to support the growth of the pathogen, absence in 25 g of the product is required at the end of the production, if the manufacturer is not able to prove that the bacterial counts in the product will not exceed the limit of 100 cfu/g throughout the shelf life.

During the period from 01.05.2011 to 30.04.2012, in Bulgaria a program for monitoring the prevalence of *L. monocytogenes* was performed. The monitoring involved three categories of RTE foods - gravad and smoked fish, soft and semi-soft cheeses and heat-treated meat products. The monitoring program was a part of the European baseline survey on the prevalence of *Listeria monocytogenes* in certain ready-to-eat foods at retail in the EU, 2010 - 2011 (Commission decision 2010/678/EC). A total of 367 samples were taken from three categories of RTE foods - packaged (not frozen) hot or cold smoked or gravad fish (2 × 122 samples, i.e. duplicates from the same batch), soft and semi-soft cheeses (63 samples) and packaged heat-treated meat products (60 samples). The samples were examined qualitatively according to ISO 11290 part 1:1996 and quantitatively according to ISO 11290 part 2:1998. From the fish products duplicate samples were taken and one of the samples was analyzed within 24 h after arrival at the laboratory, whereas the other one was kept refrigerated at the temperature indicated on the label and was examined at the end of the shelf life. The meat products and the cheese samples were tested only at the end of the shelf life. The fish samples were also analyzed for pH (according to ISO 2917:1999) and water activity (according to ISO 21807:2004) immediately after they had been received at the laboratory.

L. monocytogenes was detected in 12.3% (15/122) of the fish samples analyzed at the day of receiving and in 8.2% (10/122) of the samples at the end of the shelf life. The highest prevalence of *L. monocytogenes* was found in cold smoked fish and lower - in gravad and hot smoked fish. *L. monocytogenes* was not detected in any of the meat products and cheese samples. Levels of *L. monocytogenes* exceeding 100 cfu/g were found in three (2.5%) of the fish samples analyzed at the day of receiving at the laboratory and in one (0.8%) of the samples tested at the end of the shelf-life. The presented data were statistically processed by Student's *t*-test.

L. monocytogenes was found only in RTE fish products (15 isolates). The maximum level of 100 cfu/g was surpassed in only three of the cases.

***Key words**: Listeria monocytogenes, Ready-to-use foods (RTE), European Baseline survey.*

1. Introduction

Listeria monocytogenes is a ubiquitous organism that is widely distributed in the environment, especially in plant matter and soil. The main reservoirs of *Listeria* are soil, forage and water [1, 2, and 3]. Transmission of this pathogen to humans is mainly through consumption of contaminated food. The bacterium can be found in raw foods and in processed foods, which are contaminated after processing. Cooking at temperatures higher than 65 ^{0}C destroys *Listeria*, but the bacterium is able to multiply at temperatures as low as +2/+4 ^{0}C, which makes it common in ready-to-eat (RTE) foods with long shelf-life [4, 5]. *Listeria monocytogenes* can survive at low water activity, high salt concentration and can multiply from 1 to 45 ^{0}C. Some of the strains are able to survive for a long time under adverse environmental conditions and persist in food processing equipment. One of the reasons for this is its ability to form biofilms on many surfaces.

Over the five-year period 2005 - 2009, a significant elevation in the human cases of listeriosis was observed in six of the EU Member States [6] .The number of listeriosis cases in humans in 2012 increased slightly as compared with 2011. A statistically significant increasing trend in the European Union was observed also over the period 2008 - 2012, though only slowly increasing, along with a seasonal pattern. In 2011, a high fatality rate (17.8%) was reported among the cases. A total of 198 deaths due to listeriosis were reported in 2012, which was the highest number of fatal cases reported since 2006. *Listeria monocytogenes* was seldom detected above the legal safety limit from ready-to-eat foods at point of retail. Samples exceeding this limit were most often found in fishery products [7].

The aim of this study was to summarize the data obtained from the monitoring program performed in Bulgaria for 12 months period. The monitoring involved three categories of RTE foods at highest risk of contamination - gravad and smoked fish, soft and semi-soft cheeses and heat-treated meat products.This program was a part of the European Union baseline survey which was carried out in 2010 and 2011 according to Commission decision 2010/678/EU [8].

2. Materials and Methods

2.1 Survey structure

Food samples included in the study were collected at retail level of the four largest cities of Bulgaria - Sofia, Plovdiv, Varna and Burgas in which lives about half the population of the country. The samples were tested at the National Reference Center of Food Safety, NRL "*Listeria* and *E. coli*", National Diagnostic and Research Veterinary Institute in Sofia. The period for implementation of the monitoring was 01.05.2011 to 30.04.2012. The procedure for receiving and processing of samples was strictly followed. The food samples analyzed in our study were collected at retail level of the four largest cities of Bulgaria for the period 2011-2012 according to the sampling plan. In total, 367 samples were analyzed: 244 samples from smoked or gravad fish (122 × 2, i.e. duplicates from the same batch), 63 samples from soft and semi-soft cheeses and 60 samples from heat-treated meat-products. From each city 90 samples in total were collected. The fish samples were collected in duplicates from a same batch.

2.2 Food samples

The following three RTE food categories were sampled:

- Packaged (not frozen) gravad, hot smoked and cold smoked fish

Products had to be kept in vacuum or modified atmosphere. The fish could be sliced or unsliced. The package could be containing a whole fish, or half or a part of a fish. The skin could be present or absent.

- Soft or semi-soft cheeses

This category included soft or semi-soft cheeses (excluding fresh cheeses) made from raw, pasteurized or thermised milk of any animal species. The cheese could be ripened, smear-ripened, mould-ripened or brine-matured. It could be pre-packaged or wrapped in muslin, or repackaged at retail at the point of sale.

- Packaged heat-treated meat products

Products had to have undergone heat treatment and subsequent handling - sliced, smoked and vacuum, or modified.

This category included: cold, cooked meat products: meat products typically made with whole or large parts of anatomical or reformed structures (such as cooked sliced ham and cooked chicken fillet), sausages, pâtés. This category did not include meat products dried after heat treatment, such as jerky products and meat products heat-treated in an impermeable package which are not handled thereafter and fermented meat products, including fermented sausages.

Representative portion of 100 g was taken and cut into small pieces using an aseptic procedure for all analyzed samples. After that, test portions of 10 g (for enumeration method) and 25 g (for detection method) were taken and transferred into Stomacher bags before dilution.

2.3 Methods of analysis

2.3.1 Detection and enumeration of *Listeria monocytogenes*

The samples were examined for detection of *Listeria monocytogenes* according to ISO 11290 part 1 [9]. Subculture was carried out on ALOA Listeria agar (according to Ottaviani and Agosti) (Merck, Germany) and Oxford agar plate (Merck, Germany). The samples were examined for enumeration of *Listeria monocytogenes* according to ISO 11290 part 2 [10]. Subculture was carried out on ALOA.

2.3.2 Serotyping

One confirmed isolate per positive sample was serotyped by slide- and tube agglutination using *Listeria* antisera kit (Denka Seiken Co, LTD, Japan). The method procedure was described by Seeliger and Hohne and the protocol compiled by ANSES, EU-RL for *Listeria monocytogenes* in Paris, France. Each isolate was also serotyped molecularly by Multiplex PCR - Kerouanton *et al*. [11]. Confirmation and serotyping of all isolates was performed at NRL "*Listeria* and *E. coli*".

2.3.3 Water activity and pH

Test portions were taken from one of the duplicate fish samples that were examined on the day of arrival at

the laboratory and pH was measured according to ISO 2917:1999 [12]. Results were reported to the nearest 0.05 unit of pH.

Water activity (aw) was measured according to ISO 21807:2004 [13] Results were reported to two significant figures.

The laboratory is equipped with a pH meter CONSORT type ECFG6351101B and pH-meter SARTORIUS type PY-P10 which was used for measuring pH of fish samples at arrival at laboratory. NRL has HygroLab apparatus 3, Rotronic which was used to determine water activity.

2.3.4 Statistical analysis

Results were statistically processed by ANOVA using the Microsoft Excel software.

3. Results and Discussion

L. monocytogenes contamination of three categories of RTE foods

Cheese samples

Most of the cheese samples (54%) were classified as brine-matured cheeses. The majority of cheese samples (75%) were made from pasteurized milk, 6.3 % from thermised milk and in 18.7% of the samples the type of milk treatment was unknown. All 63 analyzed cheese samples (soft and semi-soft cheeses) showed a complete absence of *Listeria monocytogenes* in 25 g product. None of the tested samples exceeded the limit 100 cfu/g as the counts were less than 10 cfu/g. Most of the tested samples were manufactured in Bulgaria - 49 samples, and the rest were imports from other EU countries. The majority of the samples were taken from supermarkets - 37 and the rest were obtained from small markets and deli shops.

Meat samples

Most of the samples (53%) tested in the category of heat-treated meat products were classified as sausages (32/60).

All analyzed (60) heat-treated meat products showed absence of *Listeria monocytogenes* in 25 g. Samples of these products in 58 of the cases were taken from foods produced in Bulgaria, while the remaining two samples were obtained from products produced in other EU countries.

A list of the tested cheese and meat samples and the prevalence of *L. monocytogenes* in RTE foods-heat-treated meat products and soft /semi-soft cheeses tested is presented in Table 1.

Fish samples

The dominant fish species sampled was *Scomber scombrus*, which constituted 45.1% of the total number of the tested fish samples. Equal numbers of smoked and gravad fish were sampled. Most of the samples (226) were taken from products produced in Bulgaria, 12 originated from other EU countries and 6 were produced in countries outside the EU. Most of these samples were taken from supermarkets - 180, followed by those obtained from small markets - 42, deli shops -18 and street sale - 4.

The highest prevalence of *L. monocytogenes* was found in cold smoked fish (21/244) and lower - in gravad (2/244) and hot smoked fish (2/244).

Table 1. Prevalence of *L. monocytogenes* in RTE foods (heat-treated meat products and soft /semi-soft cheeses)

Ready-to-eat food (RTE) categories							
Heat-treated meat products (n = 60)				Soft and semi-soft cheeses (n = 63)			
Type of sample	Detection in 25 g		Enumeration cfu/g	Type of sample	Detection in 25 g		Enumeration cfu/g
	Presence	Absence			Presence	Absence	
Sausage	0	32	< 10 cfu/g	Brine-matured cheese	0	34	<10 cfu/g
Cold, cooked meat product	0	19	< 10 cfu/g	Mould-ripened cheese	0	7	<10 cfu/g
Cooked sliced ham	0	3	< 10 cfu/g	Otherwise ripened cheese	0	17	<10 cfu/g
Cooked meat of minced meat	0	1	< 10 cfu/g	Unknown type of cheese	0	2	<10 cfu/g
Pate	0	5	< 10 cfu/g	Otherwise ripened melted cheese	0	2	<10 cfu/g

Presence of *Listeria monocytogenes* was detected in 15 samples examined immediately after arrival at the laboratory. In one of these samples the number of *Listeria* was 1300 cfu/g, two others showed counts of 340 and 150 cfu/g, respectively and three samples had low numbers of 10, 18 and 20 cfu/g. The highest count of *Listeria monocytogenes* was found in one sample of cold smoked salmon produced in Norway.

In the remaining positive samples the counts of *L. monocytogenes* was < 10 cfu/g. Ten of the fish samples examined at the end of the shelf life showed presence of *Listeria monocytogenes*. Three of the positive samples had counts of 96 000 cfu/g, 18 cfu/g and 10 cfu/g, respectively.

Levels of *L. monocytogenes* exceeding 100 cfu/g were found in three (2.5%) of the fish samples analyzed at the day of receiving at the laboratory and in one (0.8%) of the samples tested at the end of the shelf-life.

A list of the tested fish samples and the prevalence of *L. monocytogenes* in RTE foods (smoked and gravad fish) tested are presented in Table 2.

The data for pH and water activity in fish samples measured on arrival at laboratory are present in Table 3.

Table 2. Prevalence of *L. monocytogenes* in RTE foods (smoked and gravad fish)

Ready-to-eat food (RTE) – smoked and gravad fish						
Type of sample	**Detection in 25 g samples tested at arrival n = 122**		**Detection in 25 g samples tested at the end of the shelf-life n = 122**		**Enumeration cfu/g**	
	Presence	**Absence**	**Presence**	**Absence**	**Samples tested at the day of arrival**	**Samples tested at the end of the shelf-life**
Cold smoked *Scomber scombrus*	10	27	8	29	10,18,20,340 cfu/g 33 samples - < 10 cfu/g	10,18 cfu/g 35 samples - < 10 cfu/g
Cold smoked *Clupea harengus*	0	4	0	4	< 10 cfu/g	< 10 cfu/g
Cold smoked *Sarda sarda*	0	1	0	1	< 10 cfu/g	< 10 cfu/g
Cold smoked sardine	1	0	0	1	< 10 cfu/g	< 10 cfu/g
Cold smoked herring Cold smoked salmon	0	1	0	1	150 cfu/g	< 10 cfu/g
Cold smoked *Hypophtalmichthyes molitrix*	1	0	1	0	1300 cfu/g	< 10 cfu/g
Hot smoked trout	0	1	0	1	< 10 cfu/g	< 10 cfu/g
Hot smoked *Scomber scombrus*	0	1	0	1	< 10 cfu/g	< 10 cfu/g
Hot smoked herring	1	9	1	9	< 10 cfu/g	< 10 cfu/g
Hot smoked *Spratus spratus*	0	1	0	1	< 10 cfu/g	< 10 cfu/g
Hot smoked *Clupea harengus*	0	2	0	2	< 10 cfu/g	< 10 cfu/g
Gravad *Spratus spratus*	1	30	0	31	< 10 cfu/g	< 10 cfu/g
Gravad herring	0	11	0	11	< 10 cfu/g	< 10 cfu/g
Gravad *Scomber scombrus*	0	8	0	8	< 10 cfu/g	< 10 cfu/g
Gravad *Clupea harengus*	1	7	0	8	< 10 cfu/g	< 10 cfu/g
Gravad Perciformes	0	1	0	1	< 10 cfu/g	< 10 cfu/g
Gravad *Engraulis* spp.	0	2	0	2	< 10 cfu/g	< 10 cfu/g

Table 3. pH and water activity of fish samples tested on arrival at laboratory

Fish products Subtype of sample	pH		Water activity	
	Variations	X ± Sx	Variations	X ± Sx
Cold smoked *Scomber scombrus*	5.51-6.39	5.90 ± 0.316	0.88 - 0.95	0.91 ± 0.023
Cold smoked *Clupea harengus*	5.60-6.14	5.83 ± 0.120	0.92 - 0.95	0.93 ± 0.013
Cold smoked *Sarda sarda*	5.67		0.91	
Cold smoked sardine	5.56		0.95	
Cold smoked herring	4.90		0.92	
Cold smoked salmon	6.11		0.95	
Cold smoked *Hypophtalmichthyes molitrix*	5.41		0.94	
Hot smoked trout	6.54		0.97	
Hot smoked *Scomber scombrus*	5.52-6.39	6.08 ± 0.241	0.88 - 0.98	0.95 ± 0.031
Hot smoked herring	5.92		0.96	
Hot smoked *Spratus spratus*	5.06 4.62		0.88 0.88	
Hot smoked *Clupea harengus*	5.09		0.88	
Gravad *Spratus spratus*	3.74-5.06	4.59 ± 0.288	0.88 - 0.94	0.91 ± 0.018
Gravad herring	4.23-5.91	4.77 ± 0.450	0.88 - 0.93	0.91 ± 0.017
Gravad *Scomber scombrus*	3.97-6.47	4.88 ± 0.701	0.91 - 0.96	0.92 ± 0.017
Gravad *Clupea harengus*	3.95-5.82	4.72 ± 0.497	0.89 - 0.95	0.92 ± 0.018
Gravad Perciformes	4.24		0.88	
Gravad *Engraulis* spp.	4.65 4.68		0.88 0.90	

Of the 25 food isolates (15 isolates from samples tasted after arrival at laboratory and 10 isolates from samples tasted in the end of shelf-life), 17 (68%) were identified as serotype 1/2a, molecular serogroup IIa, 4 (16%) as serotype 1/2b, molecular serogroup IIb, 2 (8%) as 1/2c, molecular serogroup IIc and 2 (8%) as serotype 4b, molecular serogroup IVb.

Fish samples were collected in duplicate to estimate the prevalence of *L. monocytogenes* at the time of purchase by consumers and to compare the values with those found after storage until the "use-by date". *L. monocytogenes* was detected in 12% of the samples analyzed after arrival at the laboratory and in 8% of the samples tested at the end of shelf life, but not in the corresponding duplicate sample in every case. This inconsistency was most likely due to an uneven distribution of *L. monocytogenes* within the same batch. Similar results were reported from corresponding studies in France and Sweden [14, 15].

The European baseline survey which was carried out in 2010 and 2011 found that, at the end of the shelf-life, the EU level prevalence of *Listeria monocytogenes* was highest in fish products (10.3%) and clearly lower in meat and cheese products: 2.1% and 0.47% respectively.

However, the proportion of samples exceeding the level of 100 cfu/g at the end of shelf-life was 1.7% for smoked and gravad fish, 0.43% for meat products and 0.06% for soft and semi-soft cheeses [16]. In New Zealand *L. monocytogenes* has been detected in a range of ready-to-eat meats; the best data are for the most commonly consumed ready-to-eat meat i.e. ham, with a prevalence of approximately 3.5% [17], fact opposite to our findings. Studies in South part of China in 2011 - 2012 proved that 10 (6.33%) of 158 retail RTE food samples were positive for *L. monocytogenes* and the contamination levels were less than 10 MPN/g, while none of 65 tested dairy products were positive for *L. monocytogenes*. The highest level of contamination of tested products was found in Cold vegetable dish in sauce and deli poultry [18], data also different from our findings. Investigation of RTE products in Estonia detected similar data to our results for fish products. Among RTE fish products, cold-smoked fish products were most frequently contaminated with *L. monocytogenes* (32.9%). Generally, the counts of *L. monocytogenes* in the tested products remained under 10 colony forming units (CFU) per gram of product. Only 2.9% and 0.8% of the RTE fish products contained L. monocytogenes in range of 100 - 1000 CFU/g and >1000 CFU/g at the end of shelf life [19]. In Israel between 1998 and 2007 were tested 10,413 samples from five groups of RTE foods: salads/dips, dairy, fish, poultry and meat. A total of 1260 isolates of *Listeria monocytogenes* were identified. The average isolation rate was 12.1% with the highest prevalence being registered in poultry (27%) [20]. Previous studies done in Bulgaria have determined presence of *L. monocytogenes* in RTE meat and milk products [21, 22, and 23].

4. Conclusions

- In conclusion we can summarize that during the last couple of years the safety of RTE meat and milk products in Bulgaria was significantly improved in respect of L. *monocytogenes* presence.

- At the same time this food-borne pathogen was detected in 12.3% (15/122) of the fish samples analyzed at the day of receiving and in 8.2% (10/122) of the samples at the end of the shelf life.

- The highest prevalence of *L. monocytogenes* was found in cold smoked fish and lower - in gravad and hot smoked fish. Levels of *L. monocytogenes* exceeding 100 cfu/g were found in three (2.5%) of the fish samples analyzed at the day of receiving at the laboratory and in one (0.8%) of the samples tested at the end of the shelf-life.

- Most of the tested fish samples had levels of water activity bellow 0.92, limit which do not stimulate the growth of *L. monocytogenes* in foods. Results for pH of the same samples were higher than 4.4 and they support the growth of *L. monocytogenes.*

- The pathogen was found only in RTE fish products (15 isolates). The maximum level of 100 cfu/g was surpassed in only three of the cases.

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