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Challenge test studies on *Listeria monocytogenes* grown in ready-to-eat dry cured pork product stored at home-simulated conditions

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Abstract

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These studies aimed to investigate the survival of *Listeria monocytogenes* on traditional dry cured sliced meat product – fillet "Elena". Two batches of sliced product were inoculated with higher and lower concentrations of *Listeria monocytogenes*, packed under air and stored at 6°C for the first batch and 6, 10 and 25°C for the second one. The storage time for each of them, at each temperature, was 20 days. The storage conditions aimed to simulate the ones, which are most probable to be encountered at consumer homes – air packing of bought products (including post-opening of vacuum packs), storage at low (chill) and high (room temperature) temperatures. Results from the first batch (high) showed a gradual decrease of *Listeria monocytogenes* concentration from 8.53 log CFU/g (day 0) to 5.53 log CFU/g, (day 20), while the second batch (low) had an increase in concentration at all three temperatures; 5.15 log CFU/g (day 0) to 7.31 log CFU/g (day 20) at 6°C, 5.15 log CFU/g (day 0) to 7.45 log CFU/g (day 20) at 10°C, and 5.15 log CFU/g (day 0) to 7.99 log CFU/g (day 20) at 25°C. The water activity before and after inoculation of the first and second batch were 0.891 and 0.906 and up to 0.906 and 0.921 respectively. Although these values act as a hurdle to prevent the growth of *Listeria monocytogenes* strains, adding 0.1 ml suspension increased the air packed slices water activity by 0.015, permitting growth on the inoculated strains of the second batch. Performing these types of challenge studies on ready-to-eat products should be an essential step for predicting the growth of pathogenic bacteria in food, in diverse storage conditions.

Keywords: meat product; *Listeria monocytogenes*; consumer; Ready-to-eat (RTE); storage conditions

Introduction

Fillet "Elena" is a traditional Bulgarian delicacy product, made from cured pork meat. The chop meat is removed from the fat, formed and salted in a special barrel. After 2-7 days, the salted meat is removed from the barrel, washed and left to dry. Once the drying period is over, the formed meat can be seasoned with a mixture of herbs. There are also variants without herbs, which often act as the cause for bacterial contamination of the meat surface. From a microbiological safety perspective, the addition of additives (nitrite, nitrate), the low water activity and pH during its ripening and maturation provide an inhospitable environment for most microbiological contaminants. Together these "hurdles" generally lead to a shelf stable-product (Holck et al., 2017).

L. monocytogenes grows between -0.4 and 50°C (Chasseignaux et al., 2002) and has the ability to grow in conditions, unsuitable for most of the pathogens in food – pH as low as 4.2 – 4.4, low storage temperature – 0-8°C, a_w 0.900 – 0.920 and high NaCl levels – up to 10%. The causal agent of human and animal listeriosis, *Listeria monocytogenes* gained in recognition as a major foodborne pathogen (Todd & Notermans, 2011) and especially in ready-to-eat (RTE) foods, in which a major control step, such as cook-

ing is missing (Juhee et al., 2014). Cooking at temperatures higher than 65°C destroys *Listeria*, but the bacterium is able to multiply at temperatures as low as +2/+4°C, which makes it common in ready-to-eat (RTE) foods with long shelf-life (Gyurova-Mehmedova et al., 2015). Being able to grow at low storage temperatures can be attributed to the intense adaptation of *L. monocytogenes*. Genes of *Listeria innocua* have been observed, that have expressed an increased ability to change their physiology during growth at temperatures extremes, both high and low, resulting in an intense increase in the stability of their permeability barrier function and thermal resistance of the cells (Bucur et al., 2018). According to a study (George et al., 1996), the minimum pH for detected growth of *L. monocytogenes* in 50 days at 20°C was 4.3 when the pH was adjusted with HCl and no growth was observed in 50 days at pH 4.0.

Contamination of cured meat products with *Listeria monocytogenes* can occur at different stages of production and retail. There can be raw material contamination during the production processes through contact with contaminated unprocessed raw materials, contaminated surfaces or contamination by the operators, handling the products in post-processing situations (Chasseignaux et al., 2002; Meloni, 2015). Previous studies on different types of products (fermented meat products and cheeses) also suggest that using starter cultures in the production of food products (Genigeorgis et al., 1991; Uyttendaele et al., 1999) will not support the growth of *Listeria monocytogenes* and products not having this characteristic will support it, and they will require additional inhibitory mechanisms to acquire similar resistance to *Listeria monocytogenes*. In the market bought Fillet "Elena", there aren't any starter cultures included (cured meat product) in its production (Lactic-acid bacteria (LAB), etc.) and because of this quality it represents an interesting product from safety perspective.

Based on another study (Anonymous, 2006), focused on consumer preferences for dry fermented sausages, the majority of consumers prefer to buy these RTE products as whole sausages, while an increasing percentage of consumer's purchase them sliced and vacuum packaged either from the processing plants or at retail points at the time of sale. Before consumption, the majority of the consumers (81.1%) repack these products in plastic (bags).

According to the European Union Food Law, for the Food Business Operator (or FBO), which is responsible for his production process and quick evaluation of hazards to human health, related to the consumption of the general populace of his product, to be able to set up the shelf life of their products they could perform microbiological challenge tests. These tests can validate the entire production process and storage of the foodstuffs (Tucci et al., 2019). Furthermore, the possibility of carrying out challenge tests is expressly provided by EC Regulation 2073/2005, which requires that, the FBO must ensure compliance with the microbiological criterion for *Listeria monocytogenes* of 100 CFU/g in food products that support its growth and also to carry out studies "to evaluate the development or survival of the microorganisms in question that may be present in the product during production and the shelf life, under reasonably foreseeable conditions of distribution and storage" (Anonymous, 2005).

The objective of the present study was to do perform challenge studies to evaluate the effect of packaging under air on the survival of *Listeria monocytogenes* by a method of post processing contamination. The storage conditions are similar to the ones that are available at most consumer homes (air packing of retail bought products; storage at refrigeration temperature; storage at room temperature) and were performed as challenge tests in the scope of EURL- Lm guidelines.

Materials and Methods

Experimental structure

For the purpose of the study, two consecutive experiments were performed. A total of 125 slices of Fillet "Elena" were purchased from retail markets. For the first experiment, a total of 35 slices were tested (retail market in Sofia, Bulgaria) and for the second one – 90 (retail market in Stara Zagora, Bulgaria). The slices for each respective experiment were from the same batch. The average weight of each slice was 3.1-3.3 grams. After purchase, the products were transported to the laboratory and stored at refrigerated temperature for no more than 24 hours before inoculation. The challenge tests lasted for 20 days, which is similar to air storage conditions in consumer refrigerators for these types of product. For the first experiment, 15 slices were inoculated with a *Listeria monocytogenes* strain (reference strain) and stored at 6°C and 15 slices were used as control samples, inoculated with sterile saline and stored again at the same temperature. For the second experiment, 45 samples, stored at 6°C, 10°C and 25°C were inoculated with *Listeria monocytogenes* strains (both reference and wild strains) and 45 samples were again inoculated with sterile saline and stored at similar temperature.

Preparation of Listeria inoculum, inoculation of samples and sample preparation

Preparation of Listeria inoculum

A suspension of three different strains of *Listeria monocytogenes* was used as an inoculum. It was comprised of

two wild strains, marked as №41, which was isolated from baked chicken fillet and №59/17, which was isolated from dry-cured formed meat sausage. The third strain was a reference strain (from EURL *Listeria monocytogenes*), labelled as 12MOB047LM. The preparation of the inoculum was carried out according to EURL, 2014 guideline (Anonymous, 2014). It states that during challenge testing for assessing growth potential of bacteria, at least 2 strains should be used, taking in account the variations in growth of the different strains. One of them should be with well-known growth characteristics. The other strains/s could be freely chosen (from foods, etc.), without the need for its growth characteristics to be known beforehand. The preparation of the inoculum was done according to EURL, 2014 (Anonymous, 2014), with two alterations – higher initial dosage of the suspension (CFU/ml) and lack of initial adaptation of the strains to the incubation temperature before their inoculation to the surface of the product. The challenge tests were done for the investigation of growth potential. The strains were stored at -80°C in Brain-Heart-Infusion Broth with added glycerol. Afterwards, they were individually revitalized on Nutrient Agar (HiMedia) for 24 h at 37°C. A single colony was cultivated in 10 ml of Soyabean-Casein Digest Medium (Casein Soyabean Digest Broth) (HiMedia) for 18h at 37°C. Following this step, there was an additional cultivation on Nutrient Agar for 24 h at 37°C. Single colonies from each plate were cultivated in sterile saline (9 ml). After serial dilutions, the target concentrations for each batch were achieved. When using multiple strains, the final suspension was prepared through the mixture of equal parts of liquid cultures of the three used strains. From each subculture 3 ml was used to achieve a 9 ml mixed suspension (Annex 7.3.2, EURL 2014). The amount of bacteria in it was confirmed according to ISO 11290-1:2017.

Inoculation of samples

The inoculation of samples was done according to EURL 2014 (Anonymous, 2014). A surface inoculation procedure was chosen, which mimics contamination during processing of samples (e.g. slicing). The samples were grouped according to the sampling days and their purpose (*Listeria monocytogenes* contaminated samples and control samples). On the surface of each slice 0.1 ml of the single strain/mixed suspension of *L. monocytogenes* was inoculated or 0.1 ml of sterile saline for the control samples. In order to achieve an even distribution of the inoculum on the surface of the sample, a sterile spreader was used. After inoculation, the samples were put in sterile stomacher bags which were then closed about 1 cm from the top and put in their respective temperatures.

Sample preparation

The samples were tested at preset days (D=days). The day of contamination of the samples was marked as D_0, while the end of the experiment was marked as D_{end}. The inoculation of samples was performed no later than 24 hours after their purchase from the market. The experiments lasted 20 days each, with 5-day interval in between sample testing – D_0, D_5, D_{10}, D_{15}, D_{20}. Each individual slice was with approximate weight of 3.3 – 3.5 grams. In order for the correct amount of sample to be taken, 3 slices were grouped into 1 sample, and this was the same for both the contaminated and control samples. The detection and enumeration of *Listeria monocytogenes* were performed in accordance to ISO 11290 – 1:2017 and ISO 11290 – 2:2017. The enumeration in contaminated samples was performed by adding 10 grams (1 sample) to 90 ml of sterile saline. The mixture was homogenized in a stomacher paddle blender (Stomacher 400 Circulator, Seward, UK). Sequential dilutions of the initial suspension were performed and inoculated at the surface of the samples. The control samples, inoculated with sterile saline, were tested for Total Microbial Count (TMC), through the plate pouring method at 30°C, according to ISO 4833-1:2013. pH was tested according to reference method ISO 2917:1999 – Meat and meat products – Measurement of pH – Reference method with pre-calibrated pH meter (Professional pH Meter PP-15, Sartorius). Water activity (a_w) testing was performed (Hygrolab C1, Rotronic, Germany) according to ISO 18787:2017 – Foodstuffs – Determination of water activity.

Statistical analysis

The statistical analysis of the study was conducted using ANOVA to analyze the differences among group means in a sample. As the ANOVA is based on the same assumption with the t test, the interest of ANOVA is on the locations of the distributions represented by means too. A p-value <0.05 indicated a significant difference.

Results

Listeria monocytogenes on Fillet "Elena" at 6°C

For the first experiment, an initial testing of the physical and chemical properties of the product showed a pH of 5.69, which was above the minimum required for the growth of *Listeria monocytogenes* pH 4.2 – 4.4; a_w of 0.891, which was below the 0.920 as minimal requirement for growth in regulation EC 2073/2005. Inoculating with 0.1 ml from the bacterial suspension on the surface of the slices increased the a_w by 0.015, up to 0.906.

The product before inoculation was also tested for natural contamination with *Listeria spp.*, and the results were negative.

Figure 1 and Table 1 summarize the results from the first experiment. The surface of the fillet was inoculated with a higher (8.53 log CFU/g) population density of *Listeria monocytogenes*. TMC of the product was measured at 5.97 log CFU/g at day 0. At day 5 and day 10 of the experiment, a decline of the population of *Listeria monocytogenes* was observed ($p < 0.01$), with 7.16 log CFU/g for day 5 and 6.44 log CFU/g for day 10 (Table 1). TMC showed an increase ($p < 0.05$) with 6.30 and 6.72 log CFU/g for day 5 and day 10 respectively. Day 15 had values of 5.71 log CFU/g for *Listeria monocytogenes* and 6.60 log CFU/g for TMC with pH of 5.46 (Table 1). At day 20, which was the end date for the experiment, a final value for *Listeria monocytogenes* was observed at 5.29 log CFU/g and TMC at 6.28. The pH values showed almost a constant curve throughout the experiment, with pH of 5.69 at the beginning of the experiment and 5.61 at its end.

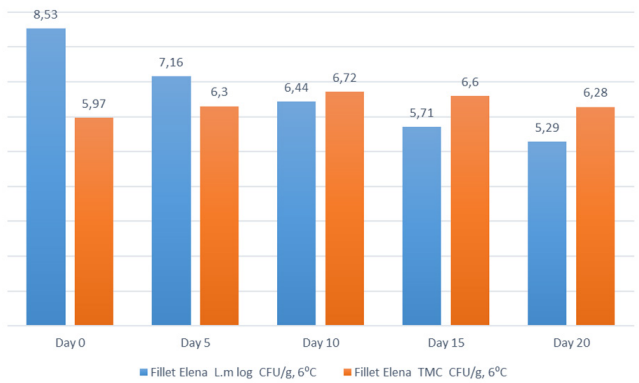


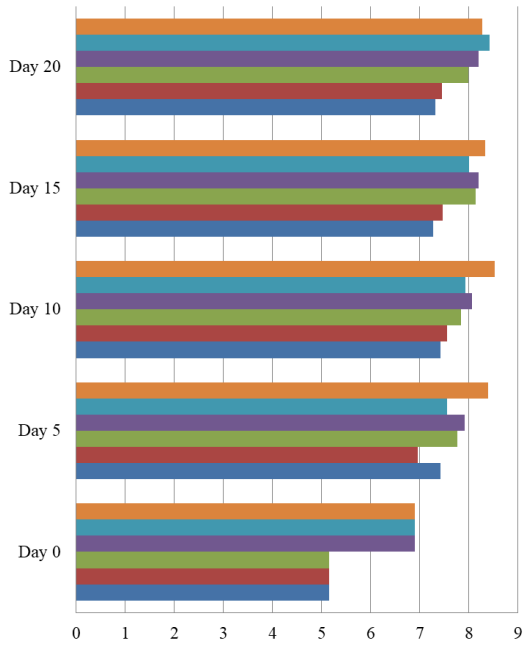
Fig. 1. Growth dynamics of *Listeria monocytogenes*, TMC and pH in Filet "Elena" during storage time for 20 days

Table. 1. Values for *Listeria monocytogenes* and TMC in Fillet "Elena" during storage time for 20 days. Legend: Statistical evaluation *($p < 0.05$);($p < 0.01$)**

Days/ Results	Fillet "Elena" *L.m* log 10 CFU/g, 6°C	Fillet "Elena" TMC log 10 CFU/g, 6°C
Day 0	8.53	5.97
Day 5	7.16	6.30
Day 10	6.44	6.72
Day 15	5.71	6.60
Day 20	5.29**	6.28*

***Listeria monocytogenes* on Fillet "Elena" at 6°C, 10°C and 25°C**

For the second experiment, the total amount of strains was expanded to include two more wild strains. Two more temperature ranges were also selected for its performance: 10°C and 25°C. Three sets of samples were prepared in sterile bags, after which they were inoculated with the strain mixture and put at their respective temperature (6°C, 10°C and 25°C). The surface of the fillet was inoculated with lower (5.15 log CFU/g) population density of *Listeria monocytogenes*. In the beginning pH was 5.71 and 5.57 at day 20. The obtained a_w value at the beginning of the experiment was 0.906. The *Listeria monocytogenes* strains grew on the food matrix, although the water activity (0.906) was below the minimum value for growth – 0.920 (EC 2073/2005). Inoculation with 0.1 ml bacterial suspension on the surface of the slices increased the water activity by 0.015, up to 0. 921. The growth of the strains showed a similar pattern at all three storage temperatures, with a rapid increase in the first 5-10 days and a "glossy" and "moist" surface inside the packs.



	Day 0	Day 5	Day 10	Day 15	Day 20
Fillet Elena TMC log CFU/g, 25°C	6,9	8,39	8,52	8,33	8,27
Fillet Elena TMC log CFU/g, 10°C	6,9	7,56	7,93	8,01	8,42
Fillet Elena TMC log CFU/g, 6°C	6,9	7,92	8,06	8,19	8,2
Fillet Elena L.m log CFU/g, 25°C	5,15	7,77	7,84	8,14	7,99
Fillet Elena L.m log CFU/g, 10°C	5,15	6,96	7,55	7,46	7,45
Fillet Elena L.m log CFU/g, 6°C	5,15	7,42	7,42	7,27	7,31

Fig. 2. Values for log for *Listeria monocytogenes* and TMC in Filet "Elena" during storage time for 20 days at 6°C, 10°C and 25°C

By inoculating the surface of the slice with 0.1 ml of suspension, the water activity of the product surface increases enough to allow growth, when the values before inoculation are close to the required minimum. Several additional batches of sliced fillet "Elena" were bought from different retail markets to test the water activity changes. They were inoculated with 0.1 ml of sterile saline on the surface of the product and measured for water activity before and after inoculation. The summarized results showed an average increase of 0.015 (with values as low as 0.013 and as high as 0.018) after inoculation. After performing this test, the growth from the second experiment can be explained.

The following results for *Listeria monocytogenes* and TMC were obtained in the span of 20 days and presented in Figure 2.

Discussion

The growth dynamics of *Listeria monocytogenes* after a post-process contamination of sliced dry-cured meat product, stored at 6, 10 and 25°C, were observed. In two separate experiments, the surface of fillet "Elena" slices were inoculated with higher (8.53 log CFU/g) and lower (5.15 log CFU/g) population density of *Listeria monocytogenes*. This is similar to a previous study (Gounadaki et al., 2007), which inoculated the surface of dry salami with low (4.6 log CFU/g; batch A) and high (6.5 log CFU/g; batch B) population density of *L. monocytogenes.* Contamination (Gombas et al., 2003) of sliced meat products with *Listeria monocytogenes* has higher prevalence in retail-sliced samples, obtained from retail markets, compared to prepack by the manufacturer. Retail-sliced RTE meat and poultry products are almost five times more likely to cause listeriosis than are prepackaged products on a per annum basis, and over four times more likely on a preserving basis (Endrikat et al., 2010). According to this study, almost 70% of the estimated deaths, included in a 2003 survey in the United States occurred from retail-sliced product that did not possess a growth inhibitor.

Listeria can grow at pH as low as 4.5 – 4.6 (Buchanan & Phillips, 1990), with this ability being reliant on temperature which is supported by the results of our experiment - a growth at favorable pH of 5.69. Growth was reported and observed at storage temperatures of 12°C, 8°C and 4°C with pH 5.0, but no growth at pH 4.5 (Gyurova-Mehmedova et al., 2015), which were similar to our results of growth at 6°C, with pH above 5.0. It is also proven that *Listeria innocua* grows faster than *Listeria monocytogenes* under most temperature conditions (Duh & Schaffner, 1993).

According to Commission Regulation (EC) 2073/2005, the limit of a_w that allows growth of *Listeria monocytogenes* in food is 0.920. A previous study (Nolan D.A et al., 1992), confirmed that the minimal a_w growth of *Listeria innocua* is between 0.904 and 0.897, but it was a specific case and it was conducted with growth media adjusted with glycerol. In our study the average a_w from both of the experiments was lower that the limit of 0.920, which is stated in the Commission Regulation (EC) 2073/2005, with a_w of 0.891 for the first experiment and 0.906 for the second experiment. Given these results, it can be suggested the product Fillet "Elena" can be included in the category as a Ready-to-Eat food unable to support the growth of *Listeria monocytogenes* (Barbuti et al., 2016). Although also suggested by our findings, caution should be advertised when storing products in packs with absence of ventilation and increased humidity on the surface, as these could be detrimental factors of enabling favorable conditions for growth of pathogenic microorganisms such as *Listeria monocytogenes*. There are other studies which focus on the increase of relative humidity in the storage conditions (Likotrafiti et al., 2013). It is found (Tian et al., 2013) that the relative humidity values significantly affected the survival of other food pathogens (*E. coli* O157:H7 and *S.* Typhimurium) on apple surfaces at temperatures of 4°C and 15°C. There is correlation (Iturriaga et al., 2003) between the combination of temperature and relative humidity and the increased ability of *Salmonella* Montevideo to attach to the surfaces of tomatoes and tomatillos. *Listeria monocytogenes* populations on intact produce survive better at higher relative humidity compared to ones at low relative humidity, with this trend being more pronounced when the product is stored at cooler temperatures (Claire et al., 2020; Likotrafiti et al., 2013).

Another factor to consider with the survivability of *Listeria monocytogenes* on Fillet "Elena" is in the form of the product in which it was purchased and tested: sliced samples. Rapid growth of foodborne pathogens, such as *Listeria monocytogenes* is less likely to occur when the product surface is intact compared to when it is sliced or cut in any form (Scallan et al., 2011; Takeuchi et al., 2000; Tian et al., 2013). Furthermore, it is known that bacterial cells become more resistant to stress factors when they are attached to the surface of a product compared with planktonically growing cells (Humphrey et al., 1997). The protective outer barrier of most intact food products could be detrimental in restricting the availability of nutrients and moisture, required for microbiological growth (Harris et al., 2003).

Regarding the growth difference of *Listeria monocytogenes* on the different batches of sliced product, some factors could be taken into consideration. In our experiments, using high inoculation level showed faster inactivation times, with rapidly decreasing levels (CFU/g) of *Listeria* on sampling days. Similar study (Gounadaki et al., 2007) showed that the

initial inoculation level affect (P<0.05) the duration and rate of the first inactivation phase of *L. monocytogenes*. More specifically, high inoculum (6.5 log CFU/g) resulted in a shorter *t*2D (faster destruction) than low inoculum (4.6 log CFU/g). Similar findings have also been reported by another team, which observed that high levels (6.8 log CFU/g) of *E. coli* O157:H7 experienced faster inactivation than low levels (3.8 log CFU/g) during drying of beef jerky that had been subjected acidic marinade (pH on meat surface, 4.23) before drying (Yoon et al., 2005). In the second experiment, the reduction of *Listeria monocytogenes* is at its highest at 25°C, showing the highest reduction (0.15 CFU/g) between two sampling days. Although this value is not that marginal, given the duration between sampling (5 days), in another study it is reported that with regard to storage temperature, the obtained results clearly demonstrated that the reduction of *L. monocytogenes* was greater at room temperature (25°C) than at 15°C and chill (5°C) temperature (Gounadaki et al., 2007). Also, it has been reported that aerobic conditions resulted in higher destruction of *L. monocytogenes* in simulated salami matrix than under vacuum at 20°C (Williams & Golden, 2001).

Conclusion

These challenge studies aimed to investigate the survival of *Listeria monocytogenes* on traditional dry cured meat product. The observed results suggest that sliced fillet "Elena" with water activity close to the minimum required, stored in air packs, which simulates the conditions present at consumer homes (especially at lower temperatures) can support the growth of *Listeria monocytogenes* on the products surface when the water activity is increased. Even though there is vacuum packed Fillet "Elena" sold at retail, the majority of the product is offered retail sliced, packed into non-vacuumed bags and stored at refrigeration temperatures in the consumer's home. These conditions could allow retail market contamination through handling of the product and potential growth of *Listeria monocytogenes* on otherwise unfavorable environment (low a_w). When performing challenge studies on dry cured products as per the EURL Lm 2014 and using the surface inoculation method, a good advice would be to dry the food matrix before storage in air or vacuum packs, as the added suspension increases the surface water activity of the products. By not allowing it to dry, the increased humidity can be a growth enabling factor on an otherwise non-growth supporting food product.

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