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Original article

SURVIVAL AND GROWTH DYNAMICS OF *LISTERIA MONOCYTOGENES* AND *SALMONELLA* SPP. ON ARTIFICIALLY CONTAMINATED COOKED READY-TO-EAT MEAT PRODUCTS

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Summary

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The aim of the study was to evaluate the potential of survival and growth dynamics of *Listeria monocytogenes* strains and *Salmonella enterica* subsp. *enterica* serovar Enteritidis and Typhimurium, inoculated artificially (individually and in mixture) on two ready-to-eat (RTE) cooked smoked meat products. For the purpose of the study 120 slices of cooked smoked sausage and 40 slices of cooked smoked loin were purchased and inoculated with the strains. Two storage temperatures were selected: 6 ^{o}C and 10 ^{o}C for 8 days. The study was performed as challenge tests in a secondary contamination scenario to investigate the presence and/or absence of pathogenic bacteria during the shelf life of the products. The inoculum levels at the start of the experiment were 4.46 $\log_{10}$ CFU/g and 2.88 $\log_{10}$ CFU/g for the *L. monocytogenes* strains at the surface of the cooked smoked loin and cooked smoked sausage respectively. Using the same inoculation method, but adding *Salmonella enterica* serovars to the mixture, the inoculum levels were 4.15 $\log_{10}$ CFU/g at the surface of the cooked smoked loin and 2.94 $\log_{10}$ CFU/g at the surface of the cooked smoked sausage. *L. monocytogenes* was detected at all sampling days on both storage temperatures. It showed an average increase by 0.5–1.0 $\log_{10}$ CFU/g on the cooked smoked sausage and by 2.0–3.3 $\log_{10}$ CFU/g for the cooked smoked pork loin for the duration of the study. *Salmonella enterica* serovars were also present at each sampling day on both storage temperatures. Typical colonies were isolated and serotyped, confirming the survival of these pathogenic bacteria. *Salmonella enterica* subsp. *enterica* serovar Typhimurium was the predominant serovar at almost every sampling day. The results from our study showed no competitive relationship in the presence of *Salmonella* and *L. monocytogenes* in contaminated meat products. The two types of microorganisms were successfully adapted and developed independently under appropriate conditions, including temperature, humidity, water activity and pH.

Key words: challenge test, *Listeria monocytogenes*, meat products, *Salmonella enterica*, shelf life

INTRODUCTION

Ready-to-eat (RTE) meat products may be source of contamination and foodborne illness by both *Salmonella* and *Listeria monocytogenes*, as evidenced by reported outbreaks and studies (CDC, 2010; Huusko *et al.*, 2017; Stephen *et al.*, 2018; Neri *et al.*, 2019). The EU One Health Zoonosis Reports (2018) outlined salmonellosis as the second most commonly reported gastrointestinal infection in humans after campylobacteriosis, and an important cause of food-borne outbreaks in the European Union. In 2018, *Salmonella* has caused 30.7% of all foodborne outbreaks (FO), which is an increase of over 20% compared to the previous year. From 1,580 *Salmonella* FO, 18.9 % were associated with meat and meat products.

Being a ubiquitous organism, *Listeria monocytogenes* is widely distributed in the environment. According to EFSA Panel on Biological Hazards (BIOHAZ) (Anonymous, 2018), there are a number of contamination routes through which *L. monocytogenes* can enter the RTE food chain. Soil and water are considered primary sources of transmission (Linke *et al.*, 2014). Regarding the food production processes, steps such as heating, curing, smoking, fermentation, etc., can reduce the pathogen loads on the RTE food at the time of consumption. Nevertheless, the effectiveness of these control steps depends on the type of food and process design. Post-processing contamination can also occur during handling. Increased handling leads to a higher probability of contamination (Angelidis & Koutsoumanis, 2006). Processing machinery, food contact surfaces and workers can all pose a risk of contamination of food products. *L. monocytogenes* has the ability to form biofilm when it is exposed to environment conditions. This results in enhanced resistance to a wide array of disinfectants and antimicrobial agents. The formation of biofilm and the increased endurance of the bacteria is the reason for one of the most important factors – post-process contamination after heating of the products.

Food Business Operators (FBOs) are responsible for the production process at facilities and/or the evaluation of potential hazards to human health, linked to the consumption of food products. Another responsibility of FBOs is the set-up of product shelf life and the performance of microbiological challenge tests. The purpose of these tests is to validate the entire production process and storage of 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 FBOs must ensure compliance with the microbiological criterion of 100 CFU/g for *L. monocytogenes* in food products that supports its growth and also to carry out studies to evaluate the development or survival of pathogenic microorganisms that may be present in the product during production and shelf life, under reasonably foreseeable conditions of distribution and storage (Anonymous, 2005).

The objective of this study was to perform challenge tests as a means to observe the growth relationship between *Listeria monocytogenes* and *Salmonella* spp. strains. Two main goals were set: i) to simulate a theoretical post-processing contamination scenario with bacteria inoculated both simultaneously and individually on the surface of slices from RTE meat products, and ii) to observe the growth/non-growth behaviour and relationships between both microorganisms on these types of product.

MATERIALS AND METHODS

Sample preparation

For the purpose of the study, two ready-to-eat, cooked smoked meat products were used. A total of 120 slices of a commonly sold cooked smoked sausage (the first product) were purchased from retail markets. The average weight of each slice was 3.1–3.3 g. The sausages were individually whole wrapped in protective wrap and packed together in vacuumed bags. After purchase, the products were transported to the laboratory and stored at refrigerated temperature for no more than 24 hours before inoculation. Right before the beginning of the experiment the vacuum bags were open and each individual sausage was cut into slices of uniform size. From the second product – cooked smoked pork loin, a total of 40 slices were used in the experiment. The product was purchased whole (non-cut), packed into a vacuum bag, from retail markets. After transportation, the packs were opened and the loin was cut aseptically into slices. The average weight of each slice after cutting was 10 g.

Preparation of L. monocytogenes inoculum

For the purpose of the study, a suspension of three *L. monocytogenes* strains was used to inoculate the surface of the products. Two of the strains were obtained from food products: the first one, labelled №41NCFS was isolated from baked chicken fillet and the second one, №59/17NCFS – from dry-cured formed meat sausage. The third strain was from the collection of EURL *Listeria monocytogenes* labelled 12MOB047LM. The EURL Lm Technical Guideline Document (Anonymous, 2014) was taken into account when carrying out the preparation of the inoculum. It stated that during challenge testing for assessing growth potential of bacteria, at least 2 strains should be used, taking in account the variations in the growth of the different strains. One of them should be with well-known growth characteristics. The other strain(s) could be freely chosen (from foods, etc.), without need for knowing its growth characteristics beforehand. Similar to our previous study (data not published), a higher initial dosage of the bacteria in the suspension was used (CFU/mL) and there was no initial adaptation of the selected strains to both incubation temperatures. All challenge tests concerning the strains of *L. monocytogenes* were performed for growth potential investigation. Both the wild and reference strains were stored at –80 ^{o}C. The used media for storage was Brain-Heart-Infusion (BHI) Broth with additionally added 20% glycerol. They were revitalised on Nutrient Agar (HiMedia, India) at 37 ^{o}C for 24 h. Cultivation was performed by using a sterile loop to transfer a single colony in 10 mL of Casein Soyabean Digest Broth (HiMedia, India) at 37 ^{o}C for 18 h. Following this step, a follow-up cultivation was performed on Nutrient Agar (HiMedia, India) at 37 ^{o}C for 24 h. From each plate, single colonies were transferred in glass test tubes with sterile saline (9 mL). Serial dilutions were performed to achieve the required concentrations. When using multiple strains, the final suspension was prepared through mixing 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, Anonymous, 2014). The real bacterial count in the suspension was confirmed according to ISO 11290-1:2017.

Preparation of Salmonella inoculum

In order to perform the challenge test studies with *Salmonella* serovars, two reference strains were used: *Salmonella enterica* subsp. *enterica* serovar Enteritidis (ATCC™ 13076) and *Salmonella enterica* subsp. *enterica* serovar Typhimurium (ATCC™ 14028). Both strains were provided by the NRL *Salmonella, Campylobacter,* staphylococii and AMR, NDRVI, BFSA, Sofia, Bulgaria. The strains were stored at –80 °C in glass test tubes of buffered peptone water (BPW) (Merck KGaA, Germany) with added 20% glycerol. Revitalisation and cultivation were performed on Nutrient Agar (HiMedia, India) at 37 °C for 24±2 h. From each plate, single colonies were transferred in vials with sterile saline (9 mL). Serial dilutions were performed to obtain the required concentrations. Since two strains of *Salmonella* were used, equal parts of liquid culture from both were mixed. The preparation of suspension mixture was similar to that of *L. monocytogenes* mixture. From each subculture, 4.5 mL were used to achieve 9 mL mixed suspension. Typical single colonies were randomly chosen from both selective media; afterwards they were cultivated on Nutrient Agar. Serotyping was performed by selecting typical colonies from the agar, first with polyvalent antisera (OA-OE) (Sifin Diagnostics GmbH), afterwards with specific somatic antisera for serovar Typhimurium (OB) (Sifin Diagnostics GmbH) and serovar Enteritidis (OD) (Sifin Diagnostics GmbH). Slide agglutination was used as a procedure prescribed by the sera manufacturer. A small amount of bacterial mass was transferred from a suspicious colony onto a slide and mixed with a drop of test reagent until a homogeneous suspension was achieved. Afterwards the results were evaluated according to the procedure, described in the information pamphlet.

Experimental design

The EURL 2014 guidelines were followed for inoculation of samples. The grouping of samples was done according to their purpose – slices inoculated with *L. monocytogenes* strains; slices with *Salmonella* strains; a mixture of the two bacterial strains and control samples. To mimic a possible contamination during post-processing handling of the product (e. g. slicing), a surface inoculation procedure was chosen. From the *L. monocytogenes* mixture, 0.1 mL was inoculated on the surface of the products. The same technique was used for the *Salmonella* strain mixture and for the *Listeria-Salmonella* combination mixture. The control samples were inoculated with 0.1 mL of sterile saline. A sterile spreader was used to achieve even surface distribution. At the beginning of the experiment, the inoculum levels were as followed: 4.46 log10 CFU/g and 2.88 log10 CFU/g for the *L. monocytogenes* strains at the surface of the cooked smoked loin and cooked smoked sausage, respectively. When mixed with the *Salmonella enterica* serovars, the inoculum levels were 4.15 log10 CFU/g and 2.94 log10 CFU/g at the surface of the cooked smoked loin and cooked smoked sausage, respectively. Afterwards, the samples were stored in sterile stomacher bags, which were sealed 1 cm from the opening end. The packed samples were placed in storage chambers at their respective storage temperature: 6 or 10 °C.

The samples were tested at preset days (D); D0 being the day of inoculation of the samples and Dend: marking the end of the experiment. To minimise the possible growth of the native microorganisms in

the products, sample inoculation was performed no later than 24 h after retail purchase. The sampling dates for each group were D0, D2, D4, D6 and D8 (Dend). The detection and enumeration of *Listeria monocytogenes* were performed according to ISO 11290 – 1/2017, and ISO 11290 – 2/2017. *Salmonella* growth confirmation was done according to ISO 6579-1:2017. Pre-enrichment was performed by placing 10 g of sample in 90 mL of BPW. Afterwards, enrichment was followed by transferring 1 mL of BPW in Muller-Kauffmann Tetrathionate Novobiocin (MKTTn) broth and 0.1 mL of BPW in Rappaport Vassiliadis (RV) broth. Selective enrichment was carried out in xylose lysine deoxycholate (XLD) agar and Rambach agar. Typical colonies were serotyped according to CD CEN ISO/TR 6579-3:2014. For *L. monocytogenes*, the enumeration of bacteria in contaminated samples was performed by adding 10 g (or 1 sample) to 90 mL of sterile saline. Afterwards, the resulting mixture was homogenised in a stomacher paddle blender (Stomacher 400 Circulator, Seward, UK). The initial sample suspension was sequentially diluted and inoculated at the surface of the agar media petri. The control samples, inoculated with sterile saline, were tested for total microbial count (TMC), through the plate pouring method at 30 ^{o}C, according to ISO 4833-1:2013.

Sample matrix pH was tested according to reference method ISO 2917:1999 with a professional pH Meter (PP-15, Sartorius). When preparing the test sample, the destructive method was used. The laboratory sample was homogenised with the appropriate equipment. The temperature was not above 25 ^{o}C. A suitable airtight container was filled with the prepared sample. Before testing, the pH-meter was calibrated using two buffer solutions (4.00 and 7.00) at the temperature of measurement. Water activity (a_w) of the matrix product was determined according to ISO 18787:2017 (Hygrolab C1, Rotronic, Germany) as per manufacturer's recommendations. The measurement was taken at 25 ^{o}C by using the water activity measurement apparatus. Calibration was performed with two standards to frame the a_w values of the tested samples.

The challenge tests lasted for 8 days in air chamber with environmental conditions similar to air storage conditions in consumers' refrigerators for these types of products. Two storage temperatures were chosen: 6 and 10 ^{o}C. Each product was sliced and divided into 8 equal groups – 4 groups at 6 ^{o}C and 4 groups at 10 ^{o}C. For cooked smoked sausage, 3 slices were grouped into 1 sample for both contaminated and sterile samples. For each temperature the cooked smoked sausage was inoculated with *L. monocytogenes* strain mixture (5×3 slices); with *Salmonella spp*. strain mixture (5×3 slices); combination of *L. monocytogenes* and *Salmonella enterica* strains (5×3 slices) and with sterile saline (5×3 slices). For the cooked smoked loin, again for both temperatures, the inoculation was performed with *L. monocytogenes* strain mixture (5 slices); with *Salmonella enterica* strain mixture (5 slices); with a combination of *L. monocytogenes* and *Salmonella* strains (5 slices) and with sterile saline (5 slices).

Statistical analysis

ANOVA was used to perform the statistical analysis of study data and to analyse the differences among group means. The used statistical software was Microsoft Excel. Differences with P value <0.05 were considered as significant.

RESULTS

Both products were tested before the start of the experiment for the presence of *Salmonella* and *L. monocytogenes* and the results came out negative. Before inoculation, the water activities were measured: the cooked smoked sausage had a_w=0.954±0.008 and the cooked smoked pork loin had a_w=0.958±0.008. The pH values were 6.45±0.002 for the cooked smoked sausage and 6.31±0.002 for the cooked smoked pork loin. Growth rates of *L. monocytogenes* strains, inoculated either individually or in combination with *Salmonella* strains on both products are presented at 6 ^oC (Table 1) and 10 ^oC (Table 2). All values obtained during the experiments were from a single sampling, without repetitions.

The surface of the cooked smoked pork loin at the start of the experiment (day 0) showed a population density of 4.46 log_{10} CFU/g for *L. monocytogenes* when inoculated alone and 4.15 log_{10} CFU/g in combination with *Salmonella* strains. The measured total bacterial count (TBC) was 6.35 log10 CFU/g at the same day. The population density at the surface of the cooked smoked sausage was 2.88 log_{10} CFU/g (*L. monocytogenes* strains only) and 2.94 log_{10} CFU/g (*L. monocytogenes* and *Salmonella* strains). The TBC was 1.26 log_{10} CFU/g.

At day 2 of the experiment at a storage temperature of 6 ^oC, the surface population density on the boiled smoked pork loin has increased to 5.59 log_{10} CFU/g (*Listeria* strains only) and 6.06 log_{10} CFU/g (*Listeria* + *Salmonella*). At a storage temperature of 10 ^oC, an increase was also reported: from 4.46 to 6.27 log_{10} CFU/g for samples inoculated only with *L. monocytogenes* strains and from 4.46 to 5.72 log_{10} CFU/g for those, contaminated with the *Listeria* and *Salmonella* bacterial mixture. The observed TBC counts were 8.48 log_{10} CFU/g at 6 ^oC and 8.49 log_{10} CFU/g at 10 ^oC storage temperatures. The surface of the cooked smoked sausage showed a decrease in population density at both storage temperatures and both variations of used inoculum. At 6 ^oC, the counts of *L. monocytogenes* decreased from 2.88 to 2.56 log_{10} CFU/g when inoculated alone and from 2.94 to 2.66 log_{10} CFU/g when in mixture.

Similar results were detected at the higher storage temperature – from 2.88 to 2.66 log_{10} CFU/g (without *Salmonella*) and from 2.94 to 2.26 log_{10} CFU/g (with *Salmonella*). The TBC at 10 ^oC showed neither an increase nor a decline, with the results remaining at 1.26 log_{10} CFU/g but this was not the matter with the results observed at 6 ^oC. At this storage temperature, the counts of bacteria increased significantly from 1.26 log_{10} CFU/g to 5.61 log_{10}CFU/g.

Day 4 and Day 6 of the experiments provided insight on a further increase in population density of *L. monocytogenes* on the cooked smoked pork loin at both temperatures. When inoculated alone, the average count increased from 6.66 log_{10} CFU/g (D4) to 7.07 log_{10} CFU/g (D6) at 6 ^oC and from 7.84 log_{10} CFU/g (D4) to 7.87 log_{10} CFU/g (D6) at 10 ^oC; in combination with *Salmonella enterica* serovars – from 6.40 log_{10} CFU/g (D4) to 6.96 log_{10} CFU/g (D6) at 6 ^oC and from 6.85 log_{10} CFU/g (D4) to 6.94 log10 CFU/g (D6) at 10 ^oC. An increase was also present in the TBC results from the same sampling days: 9.32 log10 CFU/g (D4) and 9.04 log10 CFU/g (D6) at 6 ^oC; 9.63 log10 CFU/g (D4) and 9.65 log10 CFU/g (D6) at 10^{o}C. No sampling was performed at the end of the experiment (Dend) for TBC in the cooked smoked pork loin at both temperatures, because of the already

Table 1. Growth rate of *Listeria monocytogenes* only and in mixture with *Salmonella* strains, and control total bacterial counts (TBC) at 6 °C on two food matrices (mean ± SD)

	Cooked smoked pork loin			Cooked smoked sausage		
	Listeria monocytogenes; log10 CFU/g (n=5)	*L. monocytogenes* + *Salmonella*; log10 CFU/g (n=5)	Control TBC; log10 CFU/g (n=5)	*Listeria monocytogenes*; log 10 CFU/g (n=5)	*L. monocytogenes* + *Salmonella*; log 10 CFU/g (n=5)	Control TBC; log 10 CFU/g (n=5)
Day 0	4.46±0.11	4.15±0.08	6.35±0.12	2.88±0.09	2.94±0.22	1.26±0.09
Day 2	5.59±0.13	6.06±0.09	8.48±0.24	2.56±0.25	2.66±0.81	5.61±0.36
Day 4	6.66±0.05	6.40±0.35	9.32±0.11	2.44±0.40	2.96±0.92	5.45±0.43
Day 6	7.07±0.23	6.96±0.27	9.04±0.17	3.65±1.09	3.00±0.53	6.11±0.07
Day 8	7.29±0.05	6.41±0.30		3.41±0.29	3.71±0.37	7.27±0.14

Table 2. Growth rate of *Listeria monocytogenes* only and in mixture with *Salmonella* strains, and control total bacterial counts (TBC) at 10 °C on two food matrices (mean ± SD)

	Cooked smoked pork loin			Cooked smoked sausage		
	Listeria monocytogenes; log10 CFU/g (n=5)	*L. monocytogenes* + *Salmonella*; log10 CFU/g (n=5)	Control TBC; log10 CFU/g (n=5)	*Listeria monocytogenes*; log 10 CFU/g (n=5)	*L. monocytogenes* + *Salmonella*; log 10 CFU/g (n=5)	Control TBC; log 10 CFU/g (n=5)
Day 0	4.46±0.11	4.15±0.08	6.35±0.12	2.88±0.09	2.94±0.22	1.26±0.09
Day 2	6.27±0.33	5.72±0.06	8.49±0.10	2.66±0.23	2.26±0.35	1.26±0.89
Day 4	7.84±0.62	6.85±0.13	9.63±0.22	3.59±0.34	3.24±0.78	6.03±0.39
Day 6	7.87±0.13	6.94±0.36	9.65±0.69	3.67±0.60	3.81±0.28	7.18±0.18
Day 8	7.79±0.52	6.82±0.09		3.39±0.65	4.07±0.59	8.68±0.05

high number of bacteria present in the samples and an upper limit of 9.00 log10 CFU/g for reported results. An increase in the numbers of *Listeria monocytogenes* was also present in the samples from the cooked smoked sausage: from 2.44 log10 CFU/g (D4) (a slight decrease from the value obtained at D2) to 3.65 log10 CFU/g (D6) at 6 ^{o}C (*Listeria* only) and from 2.96 log10 CFU/g (D4) to 3.00 log10 CFU/g (D6) (*Salmonella* + *Listeria*); from 3.59 log10 CFU/g (D4) to 3.67 log10 CFU/g (D6) (*Listeria* only) and from 3.24 log10 CFU/g (D4) to 3.81 log10 CFU/g (D6) (*Salmonella* + *Listeria*). The TBC results followed a similar trend towards increasing population density with a dramatic increase of more than 4.00 logs from the values obtained from the samples stored at 10 ^{o}C – from 1.26 to 6.03 log10 CFU/g (D4) and from 6.03 to 7.18 log10 CFU/g (D6). The samples stored at lower temperature followed with an initial decrease in bacterial count at D4 – 5.45 log10 CFU/g (from 5.61 log10 CFU/g by D2 to a rise of 6.11 log10 CFU/g by D6).

By day 8 of the experiment (Dend) the following final results were obtained for the cooked smoked pork samples. At the lower storage temperature, the samples inoculated only with *L. monocytogenes* strains showed increased population density of 7.29 $\log_{10}$CFU/g. Those contaminated with mixture of pathogenic bacteria showed a decline to 6.41 $\log_{10}$ CFU/g. For the higher storage temperature, both groups showed a decline in population density, with those inoculated with *Listeria* only exhibiting a reduction from 7.87 to 7.79 $\log_{10}$ CFU/g, and those contaminated with *Salmonella+Listeria*– from 6.94 to 6.82 $\log_{10}$ CFU/g. For the cooked smoked sausage, only the samples stored at 10 ^{o}C and inoculated with *Listeria* alone demonstrated a decrease in reported numbers – from 3.67 log10 CFU/g to 3.39 log10 CFU/g (Dend). The other results were as followed: for the *Listeria+Salmonella* inoculated samples at 10 ^{o}C, the final population density was 4.07 $\log_{10}$CFU/g. Those stored at 6 ^{o}C had a final population density of 3.41 log10 CFU/g and 3.71 for the samples contaminated with *Listeria* alone and with *Listeria+Salmonella,* respectively. Also, increased TBC was found out in cooked smoked sausage samples – 7.27 log10 CFU/g (6 ^{o}C) and 8.68 log10CFU/g (10 ^{o}C).

There was a statistically significant difference ($P<0.05$) between sampling day results for *L. monocytogenes* inoculated samples and *L. monocytogenes+ Salmonella enterica* inoculated samples for both products at the pre-determined storage temperatures.

To track and observe the presence of *Salmonella* on the cooked smoked food matrices, the methods, described in ISO documents (6579-1:2017; 6579-3:2014) were followed. The chosen selective media were XLD and Rambach agar. On XLD agar, black colonies (suspicious for presence of *Salmonella*) were isolated and cultivated. From Rambach agar, pink colonies (suspicious for presence of *Salmonella*) were also isolated and cultivated afterwards. These types of colonies were present on each sampling day at both temperatures and both food matrices. They were also present regardless of *Salmonella* being inoculated alone or in mixture with *L. monocytogenes*. After serological testing, the observed results showed that *S*. Typhimurium was able to adapt at all experimental conditions and was serotyped from samples obtained from each sampling day. In contrast, *S*. Enteritidis was serotyped only from the cooked smoked sausage at D0 and D2 when ino-

culated alone and at D4, D6, and D8 when in combination with *L. monocytogenes*. This serovar was absent from the cooked smoked pork loin, regardless of temperature or enrichment media.

DISCUSSION

Both products had a high water activity (a_w). High a_w provides sufficient moisture to support the growth of bacteria (pathogenic and non-pathogenic), with *Salmonella* requiring minimum a_w of 0.930–0.950 and *L. monocytogenes* requiring minimum a_w of 0.920 (Tapia *et al.* (2020). Bonardi *et al.* (2017) reported that among the physicochemical parameters considered in their study, only a_w showed an effect in reducing the probability of contamination at the end of curing, suggesting that production of soft salami characterised by high a_w values appeared to negatively impact their microbiological safety. Farakos *et al.* (2014) reported that greater survival rates of *Salmonella* serotype were found when using liquid as opposed to dry inoculum, although the majority of the used inoculum in their study was liquid (92%). In their study, Keerthirathne *et al.* (2019) found out that lower temperature protected *S*. Typhimurium from the bactericidal effect of low pH. Maximum survival was observed at 4 ^{o}C at pH 4.6, with viability lost rapidly at 37 ^{o}C at the same pH. A similar study to ours, which investigated the behaviour of both *Salmonella* and *L. monocytogenes* on slices of dry-cured ham under refrigeration for prolonged period of time, was carried out by Stollewerk *et al.* (2012). They also used a surface spreading method of the inoculum mixture, by using a Drigalsky spreader to evenly distribute 0.2 mL of inoculum until complete absorption. Other studies, following the growth dynamics of both *L. monocytogenes* and *Salmonella enterica* serovars were also carried out (Sant'Ana *et al.*, 2012a,b; Stollewerk *et al.*, 2014; Mataragas *et al.*, 2015) but the pathogenic bacteria were grown and tested separately.

On intact products, *L. monocytogenes* populations survive better at higher relative humidity compared to low relative humidity, with this trend being more pronounced when the product is stored at cooler temperatures (Likotrafiti *et al.,* 2013; Claire *et al.,* 2020). *Listeria* can also grow at pH as low as 4.5–4.6 (Buchanan & Phillips, 1990), with this ability being dependent on temperature as shown by the results of our experiment: growth at favourable pH of 6.45 and 6.31. Growth was reported and observed at storage temperatures of 12 ^{o}C, 8 ^{o}C and 4 ^{o}C with pH 5.0, but no growth at pH 4.5 (Gyurova-Mehmedova *et al.,* 2014), comparably to our results of growth at 6 ^{o}C, with pH above 5.0.

ISO 20976-1:2019(E) specifies protocols for conducting microbiological challenge tests for growth studies on vegetative and spore-forming documents in raw materials and intermediate or end products. The document suggests that when choosing strains to be used in challenge tests, priority should be given to those which are isolated from food matrices, production environment or samples from outbreaks over culture type collection, even though it is not recommended. This document also suggests that a mix of strains should be used when estimating growth potential. For this reason a mixture of two strains was chosen for inoculating the samples. *Salmonella enterica* subsp. *enterica* serovar Enteritidis and *Salmonella enterica* subsp. *enterica* serovar Typhimurium were selected for the experiments because historically *S*. Typhi-

murium has been the most frequent serotype and *S.* Enteritidis acts as a causative agent of human gastroenteritis worldwide. For each sampling day, the predominant serotype was *S.* Typhimurium. This finding coincides with a meta-analysis study, performed by Ferrari *et al.* (2019) whose aim was to evaluate the prevalence and diversity of *Salmonella* enterica serovars in animal-based foods throughout five continents. With regards to mentioned serovars in pork and pork products, *S.* Typhimurium presented a ubiquitous profile with all continents reporting it. This finding is supported by the fact that this serovar is described in outbreaks worldwide involving pork (Herikstad *et al.*, 2002; Galanis *et al.*, 2006; Hendriksen *et al.*, 2011).

The results from our study show that *Salmonella* can survive in RTE meat products after contamination, for the duration of the shelf life of the product. Combining it with *L. monocytogenes* showed no negative impact on its ability to grow, evidenced by the fact that typical colonies could be found at all temperatures for the entire duration of the study. A study of the psychrotrophy of foodborne *Salmonella* (D'Aoust, 1991), supported our findings by indicating that *Salmonella* growth had been detected at chill temperatures (4 to 5.9 ^{o}C) during extended periods of storage (10–28 days). Even though enrichment was used as part of the study to increase the amount of *Salmonella* to detectable limits, according to European Commission regulation (Anonymous, 2005), it is labelled as a food safety criteria and absence of even single colonies is mandatory. According to Fahim *et al.* (2017), the presence of *Salmonella* spp. in thermally processed foods is often attributed to inadequate sanitation, poor personal hygiene during food handling, processing, storage etc. Hassanin *et al.* (2014) conducted a study on ready-to-eat (RTE) meat products to throw light on the incidence of *E. coli* and *Salmonella* species and isolate and identify them. They collected samples from three types of RTE meat and three types of chicken sandwiches from retail stores and revealed that the incidence of *Salmonella* (with serological identification) in the three examined RTE meat samples was 40% (*S.* Typhimurium 13.3%, *S.* Dublin 13.3% and *S.* Enteritidis 13.3%), 33.3% (*S.* Typhimurium 13.3%, *S.* Dublin 13.3% and *S.* Enteritidis 6.7%) and 20% (*S.* Typhimurium 6.7% and *S.* Enteritidis 13.3%). Tareq *et al.* (2014) have also isolated *Salmonella* Typhimurium from RTE chicken product in their study indicating that post cooking contamination may contribute to the risk of infection.

Tables 1 and 2 represent the growth rate of the *Listeria monocytogenes* strains on the two products at 6 and 10 ^{o}C respectively. Introducing *Salmonella* strains to the inoculation mixture showed no negative impact on the growth of both bacteria. The growth curves were similar for the individually and simultaneously inoculated samples at each storage temperature. These results showed that under appropriate circumstances (favourable pH, a_w, etc.), *L. monocytogenes* could present a risk of contamination of RTE products that have already gone through heat treatment process. Syne *et al.* (2013) conducted a study, which showed the existence of risks for microbiological contamination of finished RTE products at an investigated processing plant. They found out that the potential for contamination from air and surfaces appeared to be the most important risk factor, affecting the microbiological quality of processed meat and meat products. When the products

were heat treated as part of their production, it was an effective way of eliminating pathogenic bacteria. Both of the cooked smoked products used in our study, underwent heat treatment as part of their production – smoking (75–90 °C for 40–80 min) and boiling/steaming at 100 °C. The internal temperature of the products had reached at least 72 °C, ensuring the microbiological safety. However, a possibility of cross-contamination with *Listeria* spp. is also likely. The bacteria may be able to survive by forming biofilms on post-cooking equipment and, by extension, the environment where niches could be formed (Wong, 1998). Another probable explanation of cross contamination and occurrence of *Listeria* spp. on equipment could be due to the movement of employees, equipment from raw meat area to packaging room, thereby resulting in contamination (Syne *et al.*, 2013). One of the aims of a study, performed by Jamali *et al.* (2013) was to determine the prevalence of *Listeria* spp. and *L. monocytogenes* in RTE foods, purchased from hypermarkets and street hawkers. They discovered that consumption of RTE foods, either purchased from hypermarkets or street hawkers posed almost equal risk of listeriosis to the consumers. Contamination with *Listeria spp*. was observed in 17.9% of obtained RTE food samples. Among them, the share of *L. monocytogenes* was 63.4%. A similar study (Aragon-Alegro *et al.*, 2008) reported that 65% of sliced cooked ham was contaminated with *Listeria spp.*, 77% of them carrying *L. monocytogenes*.

CONCLUSION

The findings of this study suggests that in a secondary contamination scenario, *Listeria monocytogenes* and *Salmonella* strains could pose a risk to consumers, with both bacteria being present for the whole duration of the shelf life of the products. Poor hygiene or improper handling of products after their production could lead to contamination of the food products. A more detailed study could be performed, including additional food matrices and usage of most probable number technique to observe the number of surviving *Salmonella* on each stage of the shelf life.

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