Food Control 137 (2022) 108959

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

From chicken to salad: Cooking salt as a potential vehicle of *Salmonella* spp. and *Listeria monocytogenes* cross-contamination

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A R T I C L E I N F O

Keywords:
Foodborne
Consumer practices
Survival
Cross-contamination
Raw meat
Read-to-eat
Low-water activity

A B S T R A C T

Epidemiological studies show that improper food handling practices at home account for a significant portion of foodborne illness cases. Mishandling of raw meat during meal preparation is one of the most frequent hazardous behaviours reported in observational research studies that potentially contributes to illness occurrence, particularly through the transfer of microbial pathogens from the raw meat to ready-to-eat (RTE) foods. This study evaluated the transfer of two major foodborne pathogens, *Salmonella enterica* and Listeria monocytogenes, from artificially contaminated chicken meat to lettuce via cooking salt (used for seasoning) during simulated domestic handling practices. Pieces of chicken breast fillets were spiked with five different loads (from ca. 1 to 5 Log CFU/g) of a multi-strain cocktail of either *S. enterica* or *L. monocytogenes.* Hands of volunteers (gloved) contaminated by handling the chicken, stirred the cooking salt that was further used to season lettuce leaves. A total of 15 events of cross-contamination (three volunteers and five bacterial loads) were tested for each pathogen. Immediately after the events, *S. enterica* was isolated from all the cooking salt samples (n = 15) and from 12 samples of seasoned lettuce; whereas *L. monocytogenes* was isolated from 13 salt samples and from all the seasoned lettuce samples (n = 15). In addition, *S. enterica* and *L. monocytogenes* were able to survive in artificially contaminated salt (with a water activity of 0.49) for, at least, 146 days and 126 days, respectively. The ability of these foodborne pathogens to survive for a long time in cooking salt, make it a good vehicle for transmission and cross-contamination if consumers do not adopt good hygiene practices when preparing meals.

1. Introduction

The incidence of illness transmitted through consumption of contaminated food is a major public health problem worldwide. Human listeriosis, although rare, is one of the most serious foodborne diseases causing hospitalization and high mortality, especially among risk groups: the elderly, immunocompromised people, and pregnant women (European Food Safety Authority & European Centre for Disease Prevention and Control, 2021). On the other hand, salmonellosis accounted for 52,702 confirmed human cases in European Union (EU) in 2020, representing the second most reported gastrointestinal infection in humans after campylobacteriosis (European Food Safety Authority & European Centre for Disease Prevention and Control, 2021).

Listeria monocytogenes and different serovars of *S. enterica* are widespread in the environment and several wild and domestic animals, particularly mammals and birds, are considered potential zoonotic reservoirs of the pathogens (Carrasco et al., 2012; Filipello et al., 2020). Poultry meat has been identified as common source and a potential vehicle of *L. monocytogenes* and *Salmonella* spp. (Capita, 2003; Goh et al., 2014), with mean levels of incidence of 19.3%; and 7.10%, respectively, in Europe (Gonçalves-Tenório et al., 2018). Schäfer et al. (2017) reported the incidence of *L. monocytogenes* on poultry breast and thigh samples of chicken as 8.6 and 44.2%, respectively, in a poultry slaughter house in Brazil; being cross-contamination during the processing steps a major source of contamination (Carrasco et al., 2012). *Listeria monocytogenes* is also a major concern due to its ability to survive for long periods in the food processing environment, increasing the risk of spreading to other surface areas, and of contamination of food products (Bolocan et al., 2015; Habimana et al., 2009). Furthermore, unlike most foodborne pathogens, *L. monocytogenes* can grow and survive in

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https://doi.org/10.1016/j.foodcont.2022.108959
Received 6 January 2022; Received in revised form 4 March 2022; Accepted 7 March 2022
Available online 13 March 2022
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conditions of high salt concentration, and most importantly, at refrigeration temperatures, conferring ability to persist and multiply in the food environment (Alves et al., 2020; Hingston et al., 2019; Shah & Bergholz, 2020).

Bacteria do not usually grow in low-water activity (a_w) foods, i.e food presenting an $a_w < 0.85$ (Young et al., 2015), such as flour, spices, nuts, nuts butters, dry powders, chocolate, etc.. Nevertheless, the survival of foodborne pathogens, such as *Salmonella* and L. *monocytogenes*, for long periods of time in this type of food products has been widely reported (Santillana Farakos et al., 2014; Igo & Schaffner, 2021; Ly et al., 2019; Nummer et al., 2012; Osaili et al., 2020; Taylor et al., 2018; Tsai, et al., 2019).

Mishandling practices and improper hygienic practices are prevalent in the kitchen and processing environments and have been reported to contribute significantly to the transmission of foodborne pathogens (Cardoso et al., 2021; Chen et al., 2001; Kusumaningrum et al., 2004; Møretrø et al., 2021). The effect of various contamination routes in the food preparation environment, including unwashed hands, cutting boards, knives, and other cooking surfaces has been studied using a variety of laboratory scenarios and has shown that cross-contamination can occur between food contact surfaces and food and between contaminated raw food and RTE food by inadequate food-handling practices (Chen et al., 2001; de Jong et al., 2008; Moore et al., 2003; Redmond & Griffith, 2003; Van Asselt et al., 2008).

Salt (sodium chloride) is widely used in food, either as a flavour enhancer or a preservative to prevent microbial spoilage, and it is generally considered a microbiologically safe product, mainly due to its low a_w. However, recently, a major recall of a salt with herb seasoning due to *Salmonella* contamination took place in various European countries (https://today.rtl.lu/news/luxembourg/a/1793850.html), demonstrating the stability of this pathogen in this type of food. In addition, the survival of *Campylobacter* spp. in kitchen salt for several hours has been established, as well as its transfer from raw chicken to cooking salt as a result of mishandling practices (Santos-Ferreira et al., 2021). In this study, the transfer of *S. enterica* and *L. monocytogenes*, from artificially contaminated chicken meat to lettuce via cooking salt (used for seasoning) was evaluated during simulated domestic handling practices. In addition, the survival of both pathogens (culturable counts) in inoculated cooking salt overtime was evaluated.

2. Materials and methods

2.1. Bacterial strains and inocula preparation

Seven L. *monocytogenes* and four *S. enterica* strains were used in this study (Table 1). This group was selected to include a genetically diverse set of strains, consisting of *S. enterica* strains belonging to different serovars and *L. monocytogenes* strains from different origins (i.e., clinical, food and food-associated environments) and serotypes. Stock cultures of the strains were stored at −80 °C in brain heart infusion (BHI) broth (Biokar Diagnostics, France) supplemented with 20% (v/v) of glycerol. For inocula preparation, *Listeria* strains were streaked onto Brain-Heart agar (BHA; Biokar Diagnostic) agar and *Salmonella* strains on Trypto-Casein Soy agar (TSA, Biokar Diagnostics), and incubated at 37 °C for 24 h. Subsequently, colonies were harvested with a sterile loop to prepare a cell suspension for each strain in 1/4 strength Ringer solution (Biokar Diagnostics) adjusted to an $OD_{600} = 1$, corresponding to ca. 10^9 colony forming units (CFU)/mL. Two bacterial cocktails containing seven strains of *L. monocytogenes* or five strains of *Salmonella* were prepared by combining equal volumes of individual cell suspensions in a sterile test tube. Decimal dilutions were further performed in Ringer solution to obtain several cocktails presenting different bacterial levels to be used in the experiments detailed below. To ascertain the real CFU/ml present in each inoculum, 0.1 mL aliquots of appropriate dilutions were spread-plated in duplicate on polymyxin acriflavine lithium chloride ceftazidime aesculin mannitol agar (PALCAM, Biokar Diagnostics) and xylose-lysine-désoxycholate (XLD, Biokar Diagnostics) agar for *L. monocytogenes* and *S. enterica*, and incubated at 37 °C for 48 h and 24 h, respectively.

2.2. Cross-contamination assays

The cross-contamination model used was previously described by Santos-Ferreira et al. (2021) to simulate events of cross-contamination from artificially contaminated raw chicken meat samples to RTE lettuce via cooking salt during a meal preparation scenario; the transmission chain considered was: artificially inoculated chicken parts – hand (gloved) – salt –lettuce (Fig. 1).

Entire chicken breasts were purchase at a local supermarket, cut into small pieces and 50 g were placed in sterile stomacher bags (BagLight PolySilk, Interscience, France). All the poultry preparation material was sterilized before use. Chicken samples were inoculated with 1 mL of different *L. monocytogenes* or *S. enterica* cocktails to a final contamination level ranging from 10^2 to 10^5 CFU/g. To enable bacterial attachment, samples were then manually mixed until total absorption of the cell suspension and stored 15 min at 4 °C. Immediately before the cross-contamination assays, lettuce leaves were washed carefully with running tap water for approximately 1 min, placed on paper to remove excess water, cut into small pieces and 50 g portions were transferred into sterile stomacher bags. At the same time, 10 g of cooking salt

Table 1
Strains used in this study.

Isolate code	Origin	Sample	Serotype	Isolation year	Geographic Isolation	Reference
L. monocytogenes						
Lm 2542	Human	Placenta	4b	2010	Portugal	Magalhães et al. (2015)
FSL J1-177	Human	NA	1/2b	1997	USA	De Jesús and Whiting (2003) Fugett et al. (2006)
FSL J1-031	Human	Blood	4a	1991	Canada	De Jesús and Whiting (2003) Fugett et al. (2006)
FSL N3-013	Food	Pate	4b	1988–99	UK	Bille and Rocourt (1996) Fugett et al. (2006)
FSL R2-499	Human	NA	1/2a	2000	USA	CDC (2000) Fugett et al. (2006)
FSL N1-227	Food	NA	4b	1988–99	USA	CDC (1998) Fugett et al. (2006)
MF4077	Food-associated Environment	NA	1/2a	NA	Norway	Møretrø et al., (2017)
Salmonella						
SLM 27C	Food	Egg shell	Typhimurium	2017	Portugal	Ferreira et al. (2020)
M2016 ETBI	Broiler	NA	Infantis	2016	Hungary	NA
775W	NA	NA	Seftenberg	NA	NA	NA
INSA	Human	NA	Enteriditidis	2017	Portugal	NA

NA – Not available.

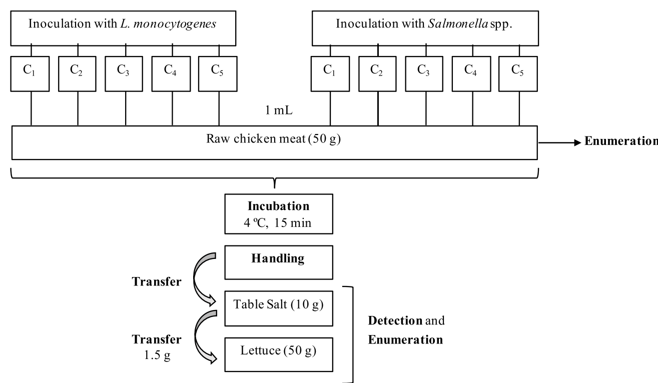

Fig. 1. Schematic representation of the transmission model of *L. monocytogenes* and *Salmonella* spp. from artificially contaminated raw chicken meat samples to lettuce seasoned with salt.

(purified sea salt in big white crystals 97.7% NaCl) were weighed into Petri dishes using an alcohol-flame sterilized spoon. For the cross-contamination assays, one volunteer touched the chicken meat inoculated with *L. monocytogenes* or *S. enterica* using powder free latex gloves (VWR Chemicals, Belgium) and then, with the same hand that touched the meat, stirred the cooking salt. Immediately after, the salt was mixed with a sterile spatula and 1.5 g, the equivalent to 1/2 teaspoon of salt (ca. 3 g), was used to season 100 g of lettuce leaves. The samples were and left undisturbed for 10 min at room temperature. Subsequently, *L. monocytogenes* and *S. enterica* detection and enumeration were performed as described below to determine contamination levels in the remaining cooking salt and lettuce. Three independent transfer experiments were performed (i.e., different days and different volunteers) for *L. monocytogenes* and *S. enterica*. Gloves were changed on each transfer assay. Uninoculated samples of chicken meat, cooking salt, and lettuce were also included to rule out possible natural contamination by *L. monocytogenes* or *S. enterica*.

2.3. Survival of L. monocytogenes and Salmonella spp. in cooking salt

The survival time of *L. monocytogenes* and *S. enterica* in cooking salt was investigated by inoculating samples of salt with multi-strain cocktails of each pathogen. Ten grams of cooking salt were aseptically weighed into sterile Petri dishes, using an alcohol flame sterilized spoon, and inoculated with five drops of 2 μl of the cocktail of each pathogen (prepared as detailed in 2.1), randomly distributed over the salt surface, to reach a final level of ca. 10^6 CFU/g. Petri dishes were maintained at room temperature (ca. 20 °C) during the experimental period to mimic the storage of cooking salt in consumer's kitchens, for several months. At selected intervals, three samples of inoculated cooking salt (i.e. three Petri dishes) were taken and bacterial enumeration was performed as described in section 2.4, specifically at times 0, 1, 6, 9, 16, 23, 38, 55, 65, 72, 93, 104, 126, 160 days for *L. monocytogenes,* and at times 0, 2, 6, 16, 31, 48, 58, 65, 80, 113, 146 and 153 days for *S. enterica.*When the numbers of bacterial cells were near to the enumeration limit, a detection method (detailed in section 2.5) was carried out simultaneously, namely at sampling times 126 and 160 days for *L. monocytogenes*, and 80, 113, 146 1st 153 days for *S. enterica*. Uninoculated cooking salt samples were included at each sampling point as a negative control. Three independent experiments were preformed per pathogen.

The a_w of the cooking salt (sea salt) used was determined at 20 °C (±0.5 °C) with a water activity meter (Novasina, Lachen, Germany), in triplicate.

2.4. Enumeration of L. monocytogenes and S. enterica

Enumeration of L. *monocytogenes* and *S. enterica* was carried out following the culture media and incubation times and temperatures recommended by the International Organization for Standardization (ISO 11290-2:2017 and ISO 6579-1:2017, respectively) with minor modifications. For the transfer assays, 50 g of chicken meat, 50 g of lettuce, or 10 g of cooking salt were placed into a sterile stomacher bag, diluted 1:10 in half-Fraser broth (bioMérieux, France) for *Listeria* or buffered peptone water (BPW; Biokar Diagnostics) for *Salmonella,* and homegenized for 2 min (BagMixer® 400, Interscience, France). Each sample was then tenfold serially diluted in Ringer, and 0.5 of the first decimal dilution and 0.1 mL of the further decimal dilutions were spread-plated onto selective agar plates for *L. monocytogenes* (PALCAM) and *S. enterica* (XLD), and incubated for 48 and 24 h at 37 °C, respectively. The enumeration limit of the method was 10 CFU/g.

For the survival assays in cooking salt, the 10 g of salt were dissolved in 1 L bottles of sterile deionized water to mitigate the effects of high salinity that could impair the bacterial growth when further plated on the solid culture media. Salt samples were manually homogenised until complete dissolution of the salt, and 0.5 and 0.1 mL aliquots were spread-plated onto non-selective (BHA or TSA) and selective (PALCAM or XLD) agar plates that were further incubated at 37 °C for 24 h and 48 h, respectively. In the last sampling points, as the bacterial numbers were decreasing, and to increase the enumeration limit, a membrane filter technique was applied. Briefly, half of the diluted samples, i.e. 0.5 L, were filtered through a 0.45 μm pore size cellulose membrane filter (Frilabo, Portugal) that was subsequently placed on the surface of the selective and non-selective agar culture media. The enumeration limit achieved was 0.2 CFU/g. Results were expressed as mean Log CFU/g of three independent replicates. *Log N/N0, N0 N.*

2.5. Detection of L. monocytogenes and S. enterica

In the transfer assays, the presence/absence of *L. monocytogenes* and *S. enterica* was determined in salt and lettuce samples using the homogenates in BPW and prepared for enumeration (in 2.4), that were further incubated at specific conditions for each pathogen (i.e., pre-enrichment step: 24 h at 30 °C for *L. monocytogenes*, and 18 h at 37 °C for *Salmonella*). In the survival assays, the detection of pathogens was ascertained when the bacterial numbers in cooking salt were below the enumeration limit; specifically, detection of *L. monocytogenes* was performed after 126 days and 160 days of storage, and of *S. enterica* after 80, 113 and 146 days of storage. For this, 0.5 L of the suspensions prepared for bacterial enumeration (in 2.4) were filtered through a 0.45 μm pore size cellulose membrane filter. The membranes were subsequently placed in a sterile stomacher bag with 90 mL of a pre-enrichment broth, i.e., half-Fraser broth (bioMérieux, Marcy-I'Étoile, France) incubated for 24 h at 30 °C for *L. monocytogenes* or BPW incubated for 20 h at 37 °C for *Salmonella*.

The following steps were executed as recommended by ISO 11290-2:2017 and ISO 6579-1:2017, for detection of *L. monocytogenes* and *S. enterica*, respectively, including species confirmation procedures. Briefly, for *L. monocytogenes* detection, following the pre-enrichment step in 90 mL of half-Fraser broth, followed by incubation for 24 h at 30 °C, a 0.1 mL aliquot was used to inoculate 10 mL of Fraser broth (Bio-Rad Laboratories, Portugal) and incubated 48 h at 37 °C. A loopful of the secondary enriched sample was plated on *Listeria* chromogenic agar base according to Ottaviani and Agosti (ALOA, bioMérieux) and PALCAM media with incubation for 24 h at 37 °C for qualitative analysis. For *S. enterica* samples, after the pre-enrichment incubation, 1 mL of the sample was transferred into 10 mL of Mueller Kauffman Tetrathionate Novobiocin Broth Base (MKTTn, bioMérieux) and 0.1 mL into 10 mL of Rappaport-Vassiliadis modified magnesium chloride/malachite green medium (RVS broth, bioMérieux) and incubated for 24 h at 37 °C and 41.5 °C, respectively. The secondary enrichment was streaked on two selective solid media (XLD and RAPID (Bio-Rad Laboratories)) and incubated for 48 h at 37 °C for qualitative analysis. Results were expressed as presence (growth) or absence (no growth) of

L. monocytogenes or *S. enterica* in 50 g of lettuce or 10 g of salt.

2.6. Molecular typing of L. monocytogenes and S. enterica isolates recovered from salt survival assays by pulsed-field gel electrophoresis (PFGE)

A selection of seven *L. monocytogenes* and 13 *S. enterica* isolates recovered during enumeration and/or detection of these pathogens at the last sampling points of cooking salt survival were typed by DNA-macrorestriction by pulsed-field gel electrophoresis (PFGE). Isolates were stored in BHI supplemented with 20% (v/v) of glycerol at - 20 °C. Typing was performed for all isolates according to the standard CDC PulseNet protocols (https://www.cdc.gov/pulsenet/pathogens/pfge.html) using the restriction enzymes AscI (New England Biolabs, Ipswich, MA, USA) for *L. monocytogenes* isolates and XbaI (Thermo Fisher Scientific, Waltham, MA) for *S. enterica* isolates. Restricted plugs were loaded into a 1% SeaKem Gold agarose gel (Lonza Group AG, Basel, Switzerland) and separated by electrophoresis in 0.5 × TBE buffer at 14 °C for 19 h), at 6 V/cm and an included angle of 120° using a Chef DR III system (Bio-Rad). *Salmonella* serotype Braenderup H9812 plugs restricted with XbaI were used as the molecular size standard. Subsequently, gels were stained using ethidium bromide solution (MP Biomedicals, Santa Ana, California, USA) and photographed using Gel Doc XR + System with Image Lab Software (Bio-Rad Laboratories). BioNumerics v.7.6.2 (Applied Maths, Sint-Martens- Latem, Belgium) was used for numerical analysis of the enzymes restriction patterns, clustered using the Dice coefficient and the unweighted pair-group method using arithmetic averages (UPGMA).

3. Results

3.1. Microbial contamination of cooking salt and its potential role as cross-contamination vehicle of foodborne pathogens

The transfer of *L. monocytogenes* and *S. enterica* from artificially contaminated raw chicken to cooking salt, via unwashed hands, and from there to seasoned lettuce are presented in Table 2 for and 3, respectively. For each pathogen, five levels of initial contamination (aiming to achieve approximately 10^1–10^5 CFU/g chicken) were tested in three independent experiments, in a total of 15 cross-contamination events. The real post-spike levels in raw chicken samples were close to what was intended and varied between 2.0×10^1 and 2.9×10^5 CFU/g, for *L. monocytogenes*, and between 8.0×10^1 and 2.6×10^5 CFU/g for *S. enterica*. As expected, higher titer experiments have led to increased bacterial loads in the cooking salt samples (up to 2.6×10^3 for *L. monocytogenes* and 4.7×10^3 for *Salmonella*), and subsequently in the lettuce samples (up to 1.3×10^3 for *L. monocytogenes* and 4.1×10^3 for *Salmonella*). Uninoculated samples of lettuce and salt included in each experiment as a control were pathogen free.

Cross-contamination of cooking salt with *L. monocytogenes*, through unwashed hands, was observed in the 13 out of 15 events, demonstrated by the detection of the pathogen in these samples (Table 2). Nine out of 15 salt samples presented contamination levels varying from 1.0×10^1 to 2.6×10^3 CFU/g, whereas six samples, spiked with the lowest levels (i.e. $< 5.0 \times 10^2$ CFU/g), presented counts below the limit of the enumeration technique (<10 CFU/g). Subsequent testing of the lettuce samples, seasoned with the potentially contaminated salt, yielded 15 samples positive for *L. monocytogenes*, and only three samples exhibited counts <10 CFU/g (corresponding to the chicken samples spiked with the lowest bacterial load in experiment III). On one occasion, the lettuce sample showed low numbers of *L. monocytogenes*, although the pathogen was not detected or quantified in the corresponding salt sample. In two events, the salt samples were positive for *L. monocytogenes*, but with counts <10 CFU/g, and the corresponding lettuce samples showed counts of 70 and 90 CFU/g.

For *S. enterica*, cross-contamination of cooking salt through unwashed hands was observed in the 15 events, regardless of the initial contamination levels of the chicken meat, as the pathogen was detected in all the samples (Table 3). Six salt samples presented counts below 10 CFU/g, specifically when chicken samples were spiked with the lowest bacterial loads in each of the three experiments, i.e. $< 1.6 \times 10^3$ CFU/g. In the lettuce samples *Salmonella* was not detected in three out of the 15 samples, specifically when chicken meat samples were contaminated with the lowest loads, i.e. $< 1.9 \times 10^2$ CFU/g. Enumeration in lettuce was only possible in five samples, when the contamination of chicken meat was above 1.9×10^4 CFU/g. *Salmonella enterica* was detected both in salt and lettuce on five occasions.

Table 2
Difference between initial *L. monocytogenes* levels on the raw chicken and final levels in cooking salt and lettuce.

Experiments	Raw chicken	Cooking salt		Lettuce	
	Counts (CFU/g)	Counts (CFU/g)	Detection (in 10 g)	Counts (CFU/g)	Detection (in 50 g)
I	2.0×10^1	<10	Negative	7.0×10^1	Positive
	3.9×10^2	<10	Positive	1.5×10^2	Positive
	1.1×10^3	7.0×10^1	Positive	2.4×10^2	Positive
	1.4×10^4	5.2×10^2	Positive	3.4×10^2	Positive
	4.8×10^4	2.6×10^3	Positive	5.1×10^2	Positive
II	1.1×10^2	<10	Positive	9.0×10^1	Positive
	5.0×10^2	1.0×10^1	Positive	6.5×10^2	Positive
	1.8×10^3	9.0×10^1	Positive	6.4×10^2	Positive
	8.0×10^3	4.9×10^2	Positive	5.1×10^2	Positive
	2.9×10^5	2.0×10^3	Positive	1.3×10^3	Positive
III	3.0×10^1	<10	Negative	<10	Positive
	1.6×10^2	<10	Positive	<10	Positive
	8.3×10^2	<10	Positive	<10	Positive
	8.7×10^3	2.0×10^2	Positive	7.0×10^1	Positive
	2.8×10^5	1.6×10^3	Positive	4.0×10^2	Positive

Table 3
Difference between initial *S. enterica* levels on the raw chicken and final levels in cooking salt and lettuce.

Experiments	Raw Chicken	Cooking salt		Lettuce	
	Counts (CFU/g)	Counts (CFU/g)	Detection (in 10 g)	Counts (CFU/g)	Detection (in 50 g)
I	9.0 × 101	<10	Positive	<10	Negative
	5.2 × 102	<10	Positive	<10	Positive
	2.7 × 103	4.0 × 101	Positive	<10	Positive
	3.3 × 104	3.3 × 102	Positive	2.8 × 102	Positive
	2.3 × 105	4.7 × 103	Positive	2.4 × 103	Positive
II	8.0 × 101	<10	Positive	<10	Negative
	1.9 × 102	<10	Positive	<10	Positive
	3.1 × 103	1.0 × 101	Positive	<10	Positive
	1.9 × 104	3.1 × 102	Positive	2.1 × 103	Positive
	1.5 × 105	1.5 × 103	Positive	4.1 × 103	Positive
III	1.9 × 102	<10	Positive	<10	Negative
	1.6 × 103	<10	Positive	<10	Positive
	8.3 × 103	4.0 × 101	Positive	<10	Positive
	3.4 × 104	4.3 × 102	Positive	<10	Positive
	2.6 × 105	1.2 × 103	Positive	2.0 × 103	Positive

3.2. Survival of L. monocytogenes and S. enterica on cooking salt

The survival of *L. monocytogenes* and *S. enterica* on cooking salt (a_w 0.49 ± 0.01) was evaluated in selective and non-selective media for five months (Fig. 2).

Salt samples were spiked with bacterial levels ranging between 4.1 and 5.6 Log_{10} CFU/g. A reduction of ca. 3 logarithmic cycles on viable counts was obtained after six and two days of exposure for *L. monocytogenes* and *S. enterica*, respectively. Throughout the storage time, the behaviour of *L. monocytogenes* was similar among the three independent experiments, and both in selective (PALCAM) and non-selective (BHA) media (Fig. 2A). For *Salmonella,* after the sixth day, a high variability was observed between selective (XLD) and non-selective media (TSA), with higher counts observed in the latter; these differences increased over time (Fig. 2B).

Inactivation of L. *monocytogenes* to levels below the enumeration limit of the method (<0.2 CFU/g) was recorded in two of the three in depend experiments after 126 days, in both media, while in one experiment the inactivation occurred after 104 (PALCAM) and 160 days (BHA; Fig. 2A). . *Salmonella enterica* was below the lower limit of the plate count assay in XLD after 16 (n = 1), 31 (n = 1), 48 days (n = 5) or 80 (n = 1) days, and in TSA after 113 (n = 5), 146 (n = 2) or 153 (n = 2) days (Fig. 2B).

When the plate counts were reaching the limit of the enumeration technique, the presence or absence of pathogens was ascertain by detection methods, namely at sampling times 126 and 160 days for *L. monocytogenes*, and 80, 113, 146 and 153 days for *S. enterica*. No L. *monocytogenes* cells were isolated from cooking salt after 104 days of storage in all the three independent replicates, while it was possible to recover *S. enterica* at times 113 and 146 days of storage.

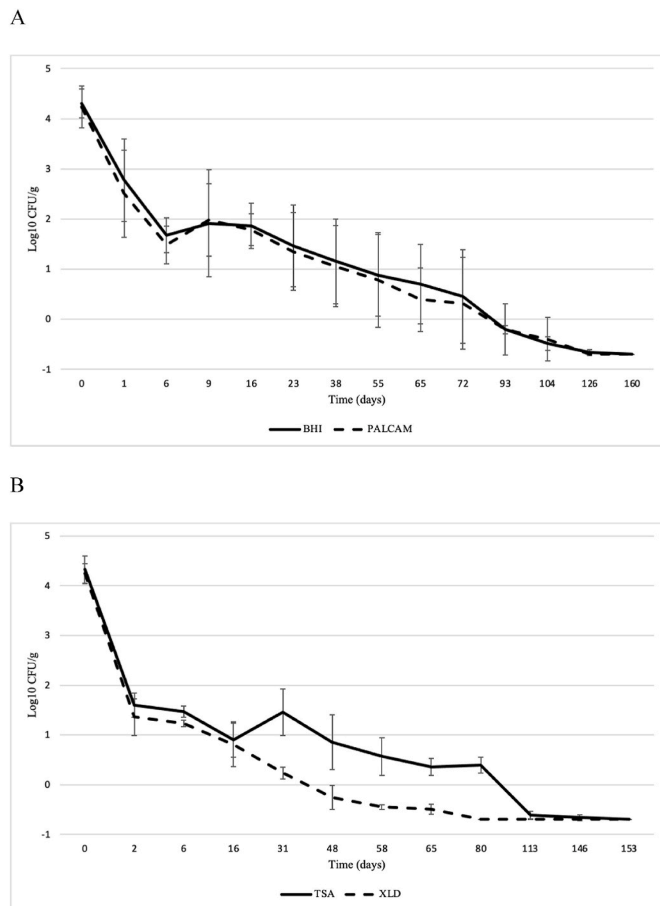

Fig. 2. Survival of L. *monocytogenes* (A) and *S. enterica* (B) in cooking salt stored at room-temperature in selective (—) and non-selective (–) media. Values represent the mean Log CFU/g of three independent replicates. The error bars show the standard deviation.

3.3. Survival of S. enterica and L. monocytogenes in cooking salt is strain dependent

Restriction analysis with PFGE was used to evaluate which strains were able to survive longer in cooking salt during storage. For that, from the last independent replicate performed, we subcultured all the colonies present in the agar plates of the enumeration method, and two randomly selected colonies of the agar plate obtained in the species confirmation step of de detection method. Overall, thirteen *Salmonella* isolates were molecular typed by PFGE (Fig. 3), including eight isolates from time 113 days (SLM1-SLM8) and five isolates from time 146 (SLM9-SLM13). Comparing the genotypes of the recovered isolates with those of the reference strains used to prepare the cocktail, it was possible to note that after 113 days of storage the four serovars were present: *S. enteriditidis* (n = 3), *S.* Senftenberg (n = 1), *S.* Typhimurium (n = 2; the two isolates from the detection method), and *S.* Infantis (n = 2). At time 146 days though, only *S.* Senftenberg (n = 2, both isolates from the detection method) and *S.* Enteriditidis (n = 1) were isolated. Concerning L. *monocytogenes,* seven isolates recovered from the enumeration method after 104 (n = 4; LMO1, LMO2, LM05, LMO6) and 126 days of storage (n = 3; LMO3, LMO4, and LM07) were typed (Fig. 4); as referred previously, no isolates were obtained by the detection method at these sampling points. Of the seven strains inoculated on salt samples, four were recovered after 104 days of storage at room temperature: FSL N1-227 (serotype 4b), FSL N3-013 (serotype 4b), Lm 2542 (serotype 4b) and MF 4077 (serotype 1/2a). At 126 days only the two latter strains were recovered, as well as strain FSL R2-499 (serotype 1/2a).

4. Discussion

Bacteria present on raw foods can be transferred to hands of food handlers during food preparation and subsequently to other surfaces handled by contaminated hands. Numerous studies indicate that a high percentage of the consumers fail to wash their hands properly after touching raw meat, with range of variation from 14% up to 90% (Evans & Redmond, 2018; Jones et al., 2017; Katiyo et al., 2019; Maughan et al., 2016). Transfer experiments carried out in this study were design to mimic improper food handling practices related to absence of hands hygiene in a domestic kitchen and to evaluate cooking salt as a new route of cross-contamination of the foodborne pathogens L. monocytogenes and *S. enterica*. After the 15 events of cross-contamination tested, enumeration of *S. enterica* was possible in nine salt samples

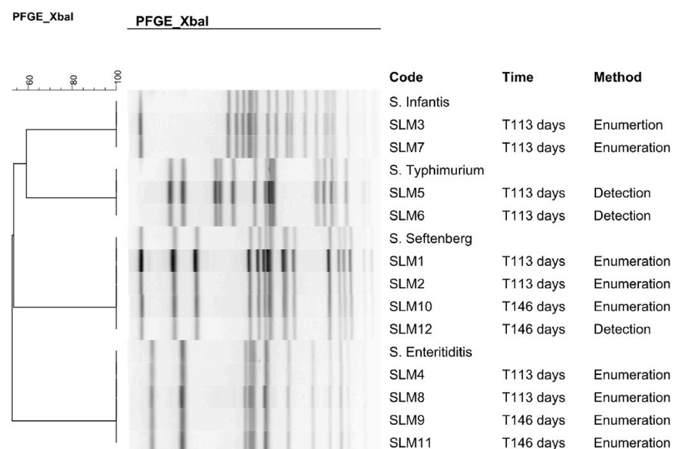

Fig. 3. Pulsed-field gel electrophoresis (PFGE) types obtained with restriction enzyme XbaI of *S. enterica* isolates collected from cooking salt samples.

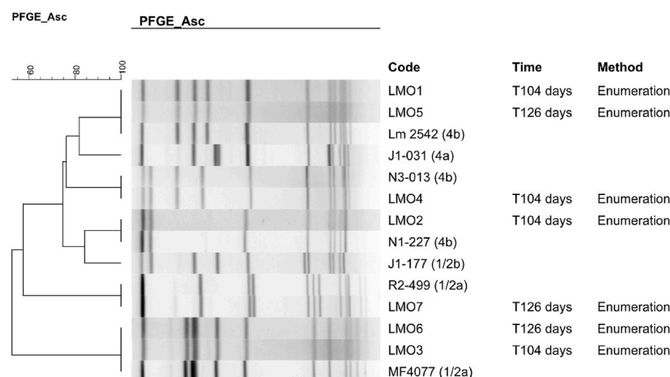

Fig. 4. Pulsed-field gel electrophoresis (PFGE) types obtained with restriction enzyme AscI of *L. monocytogenes* isolates collected from cooking salt samples.

and five lettuce samples. On five occasions it was enumerated both in salt and lettuce (initial chicken meat contamination levels ranging from 4.3 to 5.4 Log CFU/g). On the other hand, on nine occasions, *L. monocytogenes* was enumerated both in salt and lettuce, while on three occasions, the pathogen was enumerated in lettuce but not in cooking salt samples. This last scenario may be related to the random selection of salt particles to season the lettuce. Additionally, differences in the bacterial loads between the salt aliquot used to seasoned the lettuce (1.5 g) and the remaining sample (8.5 g) that was used for the detection and enumeration techniques. It is also important to note that lettuce is an inert surface that does not interfere with the survival of *L. monocytogenes*, but salt is a matrix that causes osmotic stress and damage to the cell membrane and consequent cell lysis. In addition, our results demonstrated that transfer events occur randomly and are affected by uncontrolled experimental parameters, e.g. different volunteers, contact area, duration of contact, the force applied with fingers, etc. In a real scenario, it is important to consider that raw poultry meat contains natural juices that would be transferred to the hands and then to the cooking salt jointly with the *L. monocytogenes* and *Salmonella* cells, and possibly facilitate the pathogen survival in such harsh environmental conditions.

The results of the study showed that *L. monocytogenes* and *S. enterica* can survive in cooking salt for several months. The number of *L. monocytogenes* on non-selective media (BHA) versus selective media (PALCAM) plates generally did not differ during salt exposure. On the other hand, by the detection method the pathogen was no longer detected after 104 days of storage, while bacterial counts were obtained until 126 days of storage. However, the colony numbers obtained from salt samples analysed after 104 days of storage were very low, ranging from one to three colonies; and in all the experiments all the independent assays, among the three replicates of inoculated salt samples analysed, some presented zero colonies. Furthermore, this is probably related with the enrichment steps of the ISO1129-2017 protocol in half-fraser broth followed by enrichment in full Fraser broth, that has been shown to impair the detection of stressed *L. monocytogenes* cells (Bannenberg et al., 2021).

For *Salmonella* spp. after day six of salt exposure, there was a substantial difference in recovery in non-selective (TSA) and selective (XLD) media. Non-selective media such as TSA facilitate the recovery of damaged cells because they contain vital nutrients necessary for bacterial growth. On the other hand, selective media such as XLD, favors the recovery of uninjured cells due to the inclusion of selective agents to which structurally or metabolically injured cells are susceptible. The counts were greater on TSA than on XLD, which indicates that the salt exposure caused significant cell damage. *Listeria monocytogenes* and *S. enterica* can remain viable on food contact surfaces for significant periods, increasing the risk of cross-contamination events between food handlers, food products, and food contact surfaces becoming a major cause for public health concern (Churchill et al., 2019). Despite cooking salt not being generally regarded as a food safety issue, a recent study showed the survival of *Campylobacter* spp. in this matrix for at least 4 h (Santos-Ferreira et al., 2021). The results reported here indicate that this ability to persist in sea salt is also a characteristic feature of *L. monocytogenes* and *S.* enterica, and for several weeks. The high resistance of *Salmonella* to desiccation and low a_w has been established over decades, and usually this pathogen is considered to pose a high risk in low a_w foods as it has been associated with the occurrence of numerous outbreaks and recalls (Taylor & Zhu, 2021). In previous studies, *L. monocytogenes* has demonstrated its capability to remain detectable for several months in inoculated low a_w foods, such as pistachios (a_W 0.46), almonds (a_W 0.41), and cocoa powder (a_W 0.30) (Kimber et al., 2012; Tsai et al., 2019); and it receives increased attention due to recent recalls implicating low-moisture food (Taylor & Zhu, 2021). In this study, the ability of *L. monocytogenes* strains to survive under low a_w conditions is comparable to that observed for *S. enterica* strains. Our data also suggests that this low a_w resilience is serovar- or strain-dependent. This has already been observed regarding survival of these pathogens to other environmental stresses (Alves et al., 2020; Guillén et al., 2020).

Cooking salt represents a serious cross-contamination route of foodborne pathogens in domestic settings if basic food safety practices, such as proper hand washing after handling raw meat, are not followed. A number of survey studies have reported on the unsafe practices common among consumers handling fresh products after touching raw meat or poultry (e.g., consumers do not wash their hands during meal preparation or used the same utensils/cutting boards for the preparation of both raw and ready-to-eat foods). Anderson et al. (2004) found that nearly all respondents in their study cross-contaminated raw meat, poultry and unwashed vegetables with ready-to-eat foods, and Redmond and Griffith (2003) reported that consumers used the cutting boards for the preparation of raw and RTE foods. Faour-Klingbeil et al. (2016) demonstrated the continuous transfer of pathogen from contaminated parsley to cutting boards and subsequently re-contaminating up to six batches of parsley chopped on the same board. Moore et al. (2003) showed that when a contaminated surface comes into brief contact with food, even 1–2 h after contamination, significant numbers of organisms can be transferred to the food. Ravishankar et al. (2010) showed that washing contaminated kitchen utensils in water is not sufficient to remove *S. enterica*. In an observational study carried out in the US population, the authors found that not washing hands contributed to 85% of all of the cross-contamination episodes observed (Mazengia et al., 2015). Proper handwashing was defined as washing hands with soap for a minimum of 20 s (CDC, 2017; Maughan et al., 2016). The study in the US reported that 40% of respondents wash their hands correctly after handling raw poultry (Maughan et al., 2016). Another study in Ireland reported that 76% of the respondents know they should wash their hands after handling raw meat (Moreb et al., 2017), and Myintzaw et al. (2020) reported that only 12.4% of the respondents stated that they should scrub hand for 20–30 s. Another study in China reports that only 27.2% know the correct way of washing hands (Gong et al., 2016).

The survival of *L. monocytogenes* and *S. enterica* for several months in cooking salt is worrisome as it can be used to season foods that will not be cooked, such as RTE lettuce. Furthermore, the final bacterial loads detected on lettuce were, in some occasions, higher than the pathogen's infectious dose, estimated to be less than 10 organisms for *Salmonella* (Blaser & Newman, 1982), and less than 1000 organisms for *L. monocytogenes* (Schmid-Hempel & Frank, 2007), or even lower in the case of susceptible people (e.g., the elderly; Pouillot et al., 2016).

Further studies are needed to determine the risk of listeriosis and salmonellosis under this scenario of food handling and via cooking salt.

5. Conclusion

The results from this study point to cooking salt as a potential vehicle for cross-contamination of *L. monocytogenes* and *S. enterica* from raw chicken meat to lettuce, through unwashed hands after handling artificially contaminated chicken. The detection of *L. monocytogenes* and *S. enterica* in a RTE food seasoned with contaminated salt indicates a potential risk for foodborne diseases. As observed for other low a_w foods, both pathogens were stable for several weeks in inoculated cooking salt.

Among the different causes of foodborne outbreaks, the hygiene of the kitchen and its equipment and consumer mishandling of food in the household plays an important and probably underestimates role. Therefore, a consumer's education on the risk of unsafe food preparation and handling practices is an essential element in reducing the number of viable organisms preventing listeriosis and salmonellosis.

CRediT authorship contribution statement

Ângela Alves: Methodology, Investigation, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Nânci Santos-Ferreira:** Methodology, Investigation, Visualization. **Rui Magalhães:** Methodology, Investigation. **Vânia Ferreira:** Conceptualization, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – review & editing. **Paula Teixeira:** Conceptualization, Funding acquisition, Project administration, Supervision, Validation, Visualization, All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

None.

Acknowledgements

This work was supported by SafeConsume – European Union Horizon 2020 Grant Agreement No 727580. We would also like to thank the scientific collaboration under the Fundação para a Cieˆncia e a Tecnologia (FCT) project UID/Multi/50016/2019. We thank to Martin Wiedmann from Cornell University (Ithaca, NY, USA), Trond Møretrø from NOFINA (AS, Norway), Susanne Knøchel from University of Copenhagen (Denmark), Zsuzsanna Sréterné Lancz (National Food chain Safety Office, Hungary), and Instituto Nacional de Saúde Doutor Ricardo Jorge (Lisboa, Portugal) for providing the strains.

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