Estimating *Listeria monocytogenes* growth in ready-to-eat chicken salad using a challenge test for quantitative microbial risk assessment

Bernardo, Rita[1,2]
[1] Instituto Superior Técnico (IST), Lisbon, Portugal.
[2]Centre for Interdisciplinary Research in Animal Health (CIISA), Faculty of Veterinary Medicine, Lisbon, Portugal

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Abstract

Currently, there is a growing preference for convenience foods, such as ready-to-eat (RTE) foods that are associated to long refrigerated shelf-lives and do not require a heat treatment prior to consumption. Unlike most foodborne pathogens, *Listeria monocytogenes* is able grow at refrigeration temperatures. Inconsistent temperatures during production, distribution and at consumer's household, may allow for the pathogen to thrive, reaching unsafe limits. *L. monocytogenes* is the causative agent of listeriosis, a rare but severe human illness, with high fatality rates, transmitted almost exclusively by food consumption. Therefore, it is of upmost importance to understand the behavior of *L. monocytogenes* in RTE foods. For that this study aimed to develop a challenge test in RTE chicken salads. Salads were inoculated with a three-strain-mixture of cold-adapted *L. monocytogenes* and stored at 4, 12, and 16ºC for 8 days. Results revealed the studied RTE salad was able to support *L. monocytogenes'* growth, even at refrigerated temperatures. A PMA-qPCR was used as an alternative enumeration method to the standard ISO 11290-2:2017. Throughout the study, *L. monocytogenes* isolates were detected on blank samples, and molecular characterization by multiplex PCR confirmed that the majority belonged to serogroup IVb, commonly implicated in human disease. Also, pulsed-field gel electrophoresis results suggested a persistent contamination within the assessed RTE chicken salad's producing industry. The Baranyi primary model was fitted to microbiological data to estimate the pathogen's growth kinetic parameters. Temperature effect on the maximum specific growth rate (μ_{max}) was modelled using a square-root-type model. Storage temperature significantly influenced μ_{max} of *L. monocytogenes* ($p<0.05$). These predicted growth models for *L. monocytogenes* were subsequently used to develop a quantitative microbial risk assessment, estimating an average number of 1.213×10^{-3} listeriosis cases per year linked to the consumption of these RTE salads.

Keywords: *Listeria monocytogenes*, ready-to-eat chicken salad, challenge testing, predictive growth models, genetic typing, quantitative microbial risk assessment.

1. Introduction

Listeria monocytogenes is the causative agent of a rare but severe human disease named listeriosis (Sahu et al., 2016). Listeriosis is almost entirely transmitted through the ingestion of contaminated foods, predominantly ready-to-eat foods (RTE) (Lamont, & Sobel, 2011). Listeriosis has hospitalization rates of more than 92% and a high fatality rate (between 20 and 30%), the highest of any foodborne pathogen, even with antibiotic therapy (Ajayeoba et al., 2016; EURL Lm, 2014; Melo et al., 2015). *L. monocytogenes* has the ability to adapt to very different and harsh conditions which can explain its survival and proliferation in an extensive variety of environments. *L. monocytogenes* physiological and ecological traits grant the colonization of food environments, such as processing plants, retail establishments, public and household kitchens, enabling its survival to hurdles in processing/storage, and proliferation in a variety of raw, processed and refrigerated food (Buchanan et al., 2017; Li et al., 2016; Sahu et al., 2016).

L. monocytogenes is frequently present in raw foods but can also be found as a result of cross-contamination in RTE products, such as dairy products, processed meats, salads, and other RTE foods, which do not require heating or cooking prior to consumption (Sahu et al., 2017; Takahashi et al., 2015; Uyttendaele et al., 2004). Refrigeration is one of the most common procedures used in food processing and distribution to ensure food safety during product's shelf-life (Melo et al., 2015). However, the ability of *L. monocytogenes* to grow in food stored under refrigeration temperatures, may potentially threaten consumer's health (Szczawiński et al., 2017). Because of that, European legislation establishes a limit of 100 colony-forming units per gram of RTE food at the end of shelf-life (European Commission, 2005). Food challenge testing aims to provide information on the behavior of artificially inoculated microorganisms in a food during storage under given conditions. For *L. monocytogenes,* due to its psychotropic nature, it is crucial to understand the behavior of this pathogen in ready-to-eat foods during refrigerated storage. A microbial challenge study helps food processors to ensure the safety of any food product in which potentially hazardous

microorganisms might be present in initial low numbers (Álvarez-Ordóñez et al., 2015; EURL Lm, 2014; Spanu et al., 2014).

In European member states, RTE salads have been linked to listeriosis outbreaks in the last years (Carrasco et al., 2010; Ebner et al., 2015; EFSA & ECDC, 2017; Sahu et al., 2016), therefore, accurate information on the behavior of *L. monocytogenes* in RTE salads constitutes crucial knowledge. Experimental data regarding *L. monocytogenes*' growth obtained during storage at different temperatures can be described mathematically by predictive models, which may provide useful information for the food industry and food safety regulatory agencies (Li et al., 2016), as well as be used in subsequent quantitative microbial risk assessments (QMRA) (Szczawiński et al., 2017). QMRA is often used to evaluate risks in food safety, as it offers a logical and structured approach to assess risks due to the consumption of a hazard in a specific food. This methodology has been widely applied to several RTE foodstuffs (Carrasco et al., 2010; Mataragas et al., 2010; Tirloni et al., 2018).

In the present study a stochastic QMRA model for *L. monocytogenes* was developed. The purpose of the model was to estimate the number of cases of listeriosis per year, due to the consumption of RTE chicken salads in different risk groups within the Portuguese population, and at the same time, identify the factors contributing the most to risk augmentation in the consumption of these RTE salads.

2. Materials and methods

2.1. Selection of bacterial strain

To account for variation in growth and survival among strains of *Listeria monocytogenes*, a mixture of three reference strains was used: *L. monocytogenes* CECT 4031, belonging to serogroup IIa, *L. monocytogenes* CECT 935, belonging to serogroup IVb and *L. monocytogenes* CECT 937, belonging to serogroup IIb. Additionally, these strains are representative of the three *L. monocytogenes* serogroups more frequently related to human disease, accounting for more than 95% of listeriosis cases.

2.2. Bacterial strain revival

The strains used in this study were stored at -80°C in a HERAFreeze BASIC cryogenic chamber (Thermo Fisher Scientific, Waltham, United States of America (USA)) in preservation cryotubes containing Brain Heart Infusion (BHI) broth (Scharlab, S.L., Barcelona, Spain) supplemented with 15% glycerol (Merck KGaA, Darmstadt, Germany). All procedures described subsequently were performed in Bio II Advance laminar flow chamber (Telstar Life Science solutions, Terrassa, Spain). For strains' revival, stock cultures were thawed at room temperature and 100 µl of inoculum was transferred into 5 ml of BHI broth. After 24 hours (h) of incubation at 37°C, a loop (10 µl) of inoculum was streaked onto BHI agar (Scharlab, S.L.) and incubated at 37°C for 24 h.

2.3. Preparation of *L. monocytogenes* inoculum

In order to recreate an adaptation to the refrigerated food producing-environment, the three selected *L. monocytogenes* strains were incubated separately for 4 days at 12ºC, to obtain cells in the late exponential growth phase. Cells were then centrifuged in a Centrifuge 5415 R (Eppendorf AG, Hamburg, Germany) at 6000 rpm for 10 min at 4ºC. The harvested cells were resuspended in Buffered Peptone Water (BPW) (Scharlab, S.L.) and centrifuged under the same conditions. The harvested cells of each one of the washed cultures were finally resuspended in 10 ml of BPW, and each of the *L. monocytogenes* strain' suspension was mixed together and diluted, to obtain a suspension containing approximately 10^4 cfu/ml. The inoculum density was confirmed by surface plating onto BHI.

2.4. Chicken salad production process and sample collection

In this study, a chicken salad was used. This salad was produced in a ready-to-eat food-producing industry located in an industrial park of Lisbon's metropolitan region. This salad is prepared manually in a production line located in a temperature-controlled room (10-12°C). After production, batches are stored at 5°C and have a commercial shelf-life of 6 days. In this study, samples were collected randomly from different batches produced in different weeks and transported in less than 2 hours to the laboratory in an isothermal box.

2.5. Inoculation of ready-to-eat chicken salad

For the inoculation of ready-to-eat chicken salads, random samples from each batch were chosen, and the protocol of Lokerse et al. (2016) was followed, with some adaptations. For each 100 grams of food, 1 ml of the suspension with 4 log cfu/ml of *L. monocytogenes* was inoculated and uniformly distributed with a pipette throughout the salad in the original package.

To determine specific physicochemical characteristics, as well as the concentration of commonly assessed ready-to-eat food hygiene indicators, two different blank samples were also prepared: i) blank samples inoculated with BPW, in substitution of the *L. monocytogenes* inoculum, and in the same volume as

the inoculum (BS-BPW) to test its role in the salads' microbiota, and ii) blank samples to which no PBW was added (BS), in order to detect any original contamination of the examined salads. Samples were incubated at 4ºC, 12ºC and 16ºC for 192 hours (8 days).

2.6. Food sampling

L. monocytogenes inoculated test units were analyzed at 0 h, 48 h, 96 h, 144h and 192 h (corresponding to day 0, 2, 4, 6 and 8) and blank test units were analyzed at 0 h, 96 h and 192 h. Three independent replicates (different batches) of the challenge test study were performed for each temperature.

2.7. Physicochemical analyses

For pH determination, blank samples (BS-BPW and BS) were used. Three independent measurements were performed for each homogenized sample in each sampling time point. The evaluation was done according to NP-3441 (1990), using a HI 99163 potentiometer (Hanna Instruments, Rhode Island, USA).

For a_w determination EN ISO 21807:2004 standard was used using a HygroLab C (ROTRONIC Instruments, West Sussex, United Kingdom) water activity meter with AW-40 probe, maintained at 25°C ± 2°C. For this purpose, blank sample salads (BS-BPW and BS) were used, and three independent measurements were performed for each homogenized sample in each sampling time point.

2.8. Microbiological analyses

Food samples for microbiological analyses were prepared according to ISO (International Organization for Standardization) 6887-2:2003. Microbial analyses were performed according to the respective method proposed by ISO: enumeration of total aerobic microorganisms at 30ºC (ISO 4833-1:2013), enumeration of Enterobacteriaceae (ISO 21528-2:2017), detection of *L. monocytogenes* (ISO 11290-1:2017) and enumeration of *L. monocytogenes*. Results were presented as log cfu/g.

Throughout the RTE chicken salads' challenge test, some BS revealed the presence of characteristic *L. monocytogenes* colonies on ALOA media. Confirmation of *L. monocytogenes* presumptive colonies was done by PCR.

2.9. *L. monocytogenes* DNA extraction

The isolates were grown on BHI at 37ºC for 16-18 hours, after which 200 µl of cell suspension was taken into a nuclease-free 1.5 ml microcentrifuge tube for DNA isolation, using a High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. DNA samples were stored at 4ºC. Quantification of extracted DNA was performed spectrophotometrically in a NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Fisher Scientific).

2.10. *L. monocytogenes* confirmation and serogrouping

L. monocytogenes presumptive isolates (n=30) were confirmed and serogrouped using a multiplex PCR and an additional PCR based on the amplification of the *fla*A gene (Kérouanton et al., 2010). Amplified PCR fragments were separated using electrophoresis with a 1.5% agarose gel (NZYTech) in 1x Tris borate EDTA (TBE) (Roche Diagnostics), with a 100 bp molecular weight marker (NZYTech) using GelRed (Biotium Inc., Hayward, USA) and visualized under UV light in a ChemiDoc™ XRS+ (Bio-Rad Laboratories, Hercules, USA).

2.11. Pulsed-field Gel Electrophoresis (PFGE)

DNA discs were prepared from fresh bacterial cultures on BHI plates. PFGE was performed according to the CDC PulseNet standardized procedure for typing *L. monocytogenes* (Graves, & Swaminathan, 2001). DNA was digested at 37°C for 2 h with two different macrorestriction enzymes, *ApaI* or *AscI*. Restriction fragments were separated in a 1% SeaKem Gold agarose gel, using the CHEF method in a CHEF-DRIII apparatus at 14ºC. The following electrophoresis conditions were used: voltage (6 V/cm); initial switch time, 4 s; final switch time, 40 s; run time, 19 h. Electrophoretic patterns were compared using BioNumerics® (Applied Maths, Sint-Martens-Latem, Belgium). Gels were stained in a 10 mg/ml ethidium bromide (Merck KGaA) solution for 30 min and destained one to two times with deionized sterile water for 20-30 min, after which it was visualized in a ChemiDoc™ XRS+ (Bio-Rad Laboratories).

2.12. Real Time Quantitative PCR

Samples were initially treated with propidium monoazide (PMA) (Biotium Inc.) as described previously with slight modifications (Zhang et al., 2014). DNA extraction was performed according to the guanidine thiocyanate method described from Pitcher et al., (1989), with modifications. *Listeria monocytogenes* was quantified using the commercial Genesig real-time RT-PCR (PrimerDesign™, Ltd, United Kingdom), using primers to amplify the invasion-associated protein p60 (iap) gene. Each PCR reaction incorporated 5 µl of template DNAs, ten microliters of PrecisionPLUS 2X qPCR MasterMix (PrimerDesign™, Ltd), 1 µl of *Listeria monocytogenes*-specific

primer/probe mix (detected through the FAM channel), 1 µl of internal extraction control primer/probe mix (detected through the VIC channel) and 3 µl of nuclease-free water. Genomic DNA from *L. monocytogenes* served as a positive control for the reaction and a negative control, in which the template was substituted by nuclease-free PCR grade water, was included in each run. The Applied Biosystem StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, USA) was used for *L. monocytogenes* detection and quantification according to the following cycles: first, enzyme activation at 95ºC for 2 min; second, 50 cycles of 95ºC for 10 s (denaturation) and 60ºC for 60 s (data collection). The Ct values obtained by q-RT-PCR were quantified by using a relative standard curve generated from positive control DNA at known concentrations.

2.13. Modelling growth parameters of *L. monocytogenes* in ready-to-eat chicken salads

2.13.1. Primary Model

Growth curves for each temperature were built separately by fitting data to the primary predictive model described by Baranyi and Roberts (Baranyi & Roberts, 1994) (**Equation 1-3**), using Baranyi's DMFit version 3.5 Excel® add-in (Quadram Institute). The predictive primary model was used in order to calculate the growth kinetic parameters of *L. monocytogenes* in the salads. The following parameters were obtained: 1) maximum growth rate (μ_{max}), 2) lag time (λ), 3) initial cell count (C_0) and 4) maximum population density (N_{max}).

$$\mathrm{N(t)} = N_0 + \mu_{max}A(t) - \ln\left[1 + \frac{e^{\mu max\, A(t)}-1}{e^{(N_{max}-N_0)}}\right] \quad \textbf{(1)}$$

where

$$A(t) = t + \frac{1}{\mu_{max}} ln\left(\frac{e^{(-\mu_{max}t)}+q_0}{1+q_0}\right) \quad \textbf{(2)}$$

$$\lambda = \frac{ln\left(1+\frac{1}{q_0}\right)}{\mu_{max}} \quad \textbf{(3)}$$

Where: N(t) = log of cell concentration (cfu/ml(g)) at time t (h); N_0 = log of initial cell concentration (cfu/ml(g)); μ_{max} = maximum specific growth rate (log cfu/ml(g)/h); N_{max} = log of maximum cell concentration; q_0 = parameter expressing the physiological state of cells when $t=t_0$; λ = lag time (h). In this work μ_{max} is based on the inflection of the slope of the growth curve in the exponential phase (Baranyi et al., 1993).

2.13.2. Secondary model for maximum specific growth rate

The predictive secondary model was built using the square root model described by Ratkowsky et al. (1982) (**Equation 4**) to describe μ_{max} as a function of storage temperature (T).

$$\sqrt{\mu_{max}} = b(T - T_{min}) \quad \textbf{(4)}$$

Where: μ_{max} = maximum specific growth rate (log cfu/g/h); b = regression parameter determined during the modelling process; T = storage temperature (°C); and T_{min} = determined minimum temperature for the growth of microorganisms (°C).

2.14. Statistical analyses

All microbiological and physicochemical data were assessed in a database created in GraphPad Prism 5 (GraphPad Software), using a descriptive statistical analysis with average and standard deviation calculation for the three replicates of the assays, corresponding to three batches (replicates).

For comparison of the two quantification methods (VCC and RT-qPCR), a t-test for paired samples was performed, for each temperature, and type of sample. Comparisons were performed using GraphPad Prism 5 (GraphPad Software).

A dendrogram was constructed based on PFGE patterns of the selected strains using BioNumerics software package version 6.10 (Applied Maths, Sint-Martens-Latem, Belgium). *L. monocytogenes* PFGE patterns were analyzed to determine strain relatedness with an optimization setting and a band-position tolerance of 1.5% for *Asc*I and *Apa*I restriction. Cluster analysis was performed using the unweighted pair group method with arithmetic averages (UPGMA) and band-based Dice correlation coefficient.

The estimated μ_{max} values of *L. monocytogenes* in RTE chicken salads at each isothermal storage condition were compared and checked for significant statistical differences (p-value ≤ 0.05), employing one-factor ANOVA, followed by Tukey's multiple comparison test. Statistical analyses were carried out in GraphPad Prism 5 (GraphPad Software).

2.15. Quantitative microbial risk assessment

In this study, a Monte Carlo quantitative microbiological risk assessment (QMRA) of *L. monocytogenes* in RTE chicken salads produced in a Lisbon's RTE food industry was developed. The model estimates the number of listeriosis cases linked to the consumption of RTE chicken salads. The QMRA model, considers 3 main modules:

Exposure assessment: **(a)** Prevalence and initial contamination (retail storage): at this initial stage if the product is contaminated, it will have a certain level of pathogen at that point in time. **(b)** Consumer storage: the pathogen could increase during storage depending on the consumer storage conditions, potentially leading to an increase in the final level of *L. monocytogenes* in

the product. **(c)** Consumer consumption: consumers will invariably eat different quantities (population variability) of the product, resulting in varying degrees of exposure.

Dose response: The dose response relates the amount consumed to a clinical outcome, in this study a listeriosis illness, and is used to translate exposure into a log probability of illness.

Risk characterization: This stage combines the first two steps to characterize the risk of illness for the given simulated exposure level. The model simulated the annual risk of illness for a high risk and low risk population. Each module was modelled with each proceeding module acting as an input into the next. The model provides a baseline description of listeriosis threat by consuming RTE chicken salads contaminated with *L. monocytogenes*.

The model was built using R programming language (Version 3.5.1, R Development Core Team, 2018). Overview of the model and the parameters with their values and/or distributions, and source are shown in Annex.

2.15.1. Sensitivity analysis

Sensitivity analysis is a tool that allows determining the effects that inputs have on model outputs. A sensitivity analysis was performed for the risk of listeriosis per dose. The inputs selected for the analysis were: initial concentration of the pathogen, portion size, storage temperature and time temperature. The sensitivity analysis method was implemented in R software version 3.5.1 (R Development Core Team, 2018), package: "sensitivity" (Saltelli, 2002).

3. Results and discussion

3.1. pH and a_w measurements

In **Table 1** the obtained pH and a_w values in RTE chicken salad blank samples (BS – no BWP added, and BS-BPW – BWP added) are presented, throughout the 8 days of study at the three tested temperatures: 4°C, 12°C**,** and 16°C.

Table 1. Mean and standard deviation for pH and a_w values obtained from RTE chicken salads at 4º, 12º and 16ºC throughout the challenge test (192 hours).

BS

	4°C		12°C		16°C	
Time	**pH**	**a_w**	**pH**	**a_w**	**pH**	**a_w**
0 h	5.930 ±0.033	0.955 ±0.001	6.113 ±0.191	0.957 ±0.006	6.251 ±0.561	0.962 ±0.009
96 h	6.021 ±0.080	0.966 ±0.001	6.427 ±0.400	0.957 ±0.002	6.632 ±0.554	0.971 ±0.008
192 h	6.647 ±0.491	0.980 ±0.002	7.309 ±0.325	0.958 ±0.001	7.625 ±0.167	0.963 ±0.007

BS-BPW

	4°C		12°C		16°C	
Time	**pH**	**a_w**	**pH**	**a_w**	**pH**	**a_w**
0 h	5.902 ±0.084	0.958 ±0.001	6.113 ±0.151	0.972 ±0.021	5.890 ±0.308	0.954 ±0.002
96 h	6.027 ±0.064	0.967 ±0.009	6.478 ±0.090	0.957 ±0.006	6.687 ±0.528	0.958 ±0.023
192 h	6.659 ±0.256	0.966 ±0.001	7.208 ±0.364	0.960 ±0.005	7.205 ±0.460	0.961 ±0.007

Results of pH and a_w confirmed this study's RTE chicken salad as a food product able to support the growth of *L. monocytogenes*, allowing for a precise use of the microbiological criteria limits established in both European regulation 2073/2005 and in Technical guidance document for conducting shelf-life studies on *L. monocytogenes* in foods (EURL Lm, 2014).

3.2. Hygiene indicators

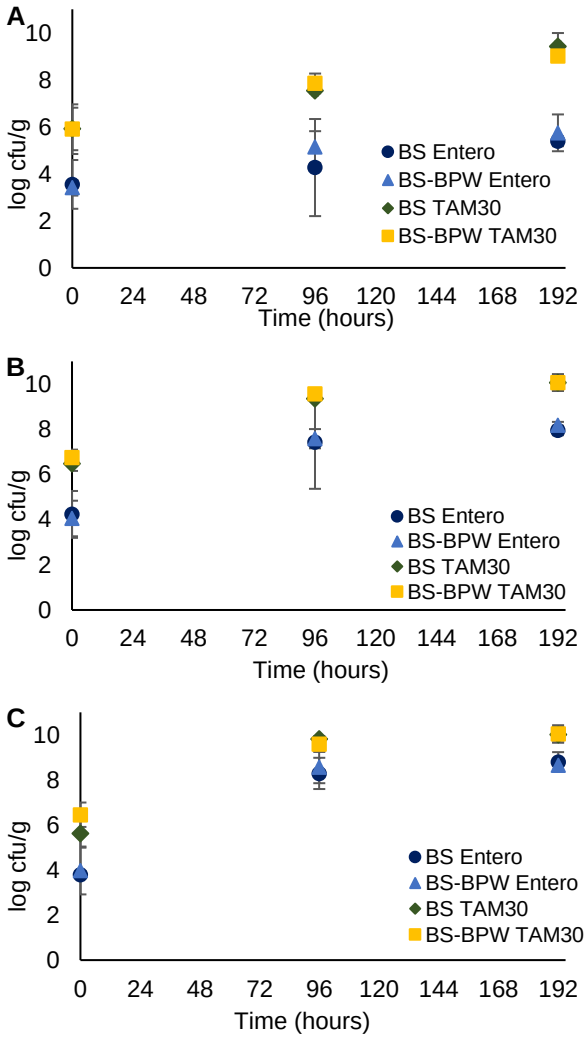

Figure 1. Mean and standard deviation (error bars) of Enterobacteriaceae (Entero) and total aerobic microorganisms at 30ºC (TAM30) viable cell counts in blank samples (BS and BS-BPW) throughout the study (192 hours). **(A)** Incubation at 4°C, **(B)** 12°C and **(C)** 16°C.

At 4ºC, for both BS and BS-BPW, on day 0, Enterobacteriaceae counts were approximately 3.5 log cfu/g, reaching 5.5 log cfu/g by the end of the studied

storage period (day 8). At 12ºC and 16ºC, initial values (day 0) were around 4 log cfu/ml for both BS and BS-BPW, reaching values of nearly 8 log cfu/g and 9 log cfu/g for 12ºC and 16ºC, respectively, at the end of storage time (**Figure 1**). These findings were in agreement with a previous study by Manios et al. (2013), where an increase in Enterobacteriaceae at 8ºC was observed on vegetable salads artificially inoculated with *L. monocytogenes*, after 10 to 12 days of storage.

At 4ºC, for both BS and BS-BPW, on day 0, the TAM30 countings were approximately 6 log cfu/g, reaching approximately 9 log cfu/g by the end of the storage period (day 8). At 12ºC and 16ºC initial values (day 0) were around 6.5 log cfu/ml for both BS and BS-BPW (except for BS at 12ºC, which was around 5.5 log cfu/g), reaching values of nearly 10 cfu/g by the end of storage time (**Figure 1**). These findings were in agreement with a previous study by Omac et al. (2018), where total aerobic microorganism' growth on fresh spinach leaves inoculated with *L. monocytogenes*, at 3ºC, 5ºC and 8ºC was also reported, after 16 days of storage. Skalina, & Nikolajeva (2010) also found a significant increase in total aerobic microorganisms on RTE mixed salads artificially inoculated with *L. monocytogenes*, throughout 48 hours of storage at 3ºC and 7ºC.

3.3. Detection and enumeration of *L. monocytogenes* on blank samples

Throughout the challenge test, it was possible to detect presumptive *L. monocytogenes* colonies in some uninoculated blank samples (BS). **Figure 2** shows an example of those colonies in ALOA plates and the subsequent isolation of some of the suspicious colonies.

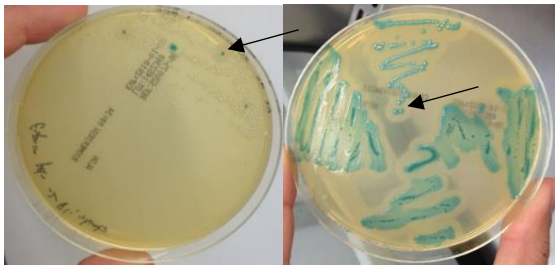

Figure 2. *Listeria monocytogenes* presumptive colonies in ALOA plates with the characteristic bluish-green color with an opaque round halo (black arrow), obtained in non-inoculated samples. The second image corresponds to the isolation of some of those colonies, also presenting the same characteristic (black arrow).

Throughout the study, these presumptive colonies (n=30) were recovered from 15 blank samples (55.6% of presumptive positive samples) on a routine basis, to be confirmed as *L. monocytogenes* by PCR (section **3.4**).

3.4. Multiplex PCR

Presumptive *L. monocytogenes* isolates' confirmation and serogrouping (n=30) was performed using a multiplex PCR, which enabled the confirmation of presumptive *L. monocytogenes* (n=10). All five molecular serogroups (IIa, IIb, IIc, Iva and IVb) were also confirmed by *L. monocytogenes* serogroups positive controls (**Figure 3**).

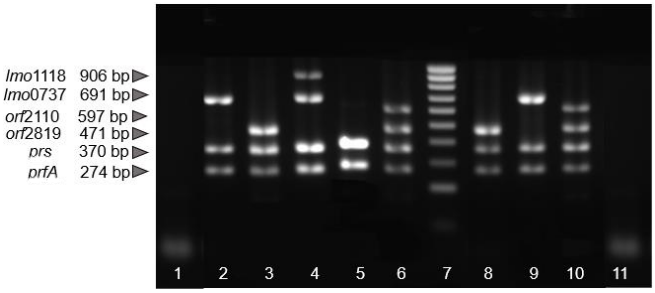

Figure 3. PCR patterns of the five molecular serogroups obtained after agarose gel electrophoresis of DNA products generated by multiplex PCR. Lane 1 - Blank reaction control; Lanes 2 to 6 - *L. monocytogenes* serogroups positive controls: Lane 2 - *L. monocytogenes* CECT4031 IIa serogroup; Lane 3 - *L. monocytogenes* CECT937 IIb serogroup; Lane 4 - *L. monocytogenes* CECT911 IIc serogroup; Lane 5 - *L. monocytogenes* CECT934 IVa serogroup; Lane 6 - *L. monocytogenes* CECT935 IVb serogroup; Lane 7 - DNA molecular weight marker (100 bp NZYTech V); Lane 8 to 10 - *L. monocytogenes* isolates in test: Lane 8 - CS1/8; Lane 9 - CS1/0; Lane 10- CS3/0-A; Lane 11- Negative control sample (*Escherichia coli* DSMZ 682).

Table 2. Serogroups of *Listeria monocytogenes* isolated from RTE chicken salads blank samples.

***L. monocytogenes* serogroup**	**Isolate code**
IIa	CS1/0
IIb	CS1/8
IVb	CS3/0-A, CS3/0-B, CS3/4-A1, CS3/4-A2, CS3/4-A3, CS5/4-A, CS5/4-B, CS7/8-A2

All the isolates not present in **Table 2**, (20 out of the initial 30 isolates) were confirmed to belong to *Listeria* genus but were not from *L. monocytogenes* species.

Three different serogroups were detected according to the presence of a specific gene distribution: IVb, IIa and IIb, representing 80%, 10% and 10% respectively (**Table 2**). It is noteworthy that *L. monocytogenes* isolates in food samples presented the 3 serogroups more implicated in human disease, namely IIa, IIb and IVb. The majority of *L. monocytogenes* isolates belonged to serogroup IVb. Isolates from serogroup IVb have been associated to the majority of clinical strains causing severe human infections (Maury et al., 2016). Moreover, during 2010 and 2012, most of human listeriosis deadly cases in Europe were linked to serogroups IIa and IVb (ECDC, 2015), and according to "*Listeria monocytogenes* contamination of ready-to-eat foods and the risk for human health in the EU" (EFSA and ECDC, 2017) the number of serogroup IVb reported cases appears to be increasing. Similar

studies are in agreement with this study's results in terms of serogroup IVb, as it was the predominant serogroup found in RTE foods (Amajoud et al., 2018; Maćkiw et al., 2016).

3.5. PFGE typing

The confirmed *L. monocytogenes* isolates were subjected to PFGE typing, to check for strain relatedness and discard any experimental contamination of the blank salad samples, for that reference strains used in the inoculation mix were included in the PFGE. Simultaneously, it intended to assess if there was a common source of contamination of the RTE chicken salads in the producing industry. The resulting dendrogram obtained from the analysis of the restriction profiles of *L. monocytogenes* isolates with *Apa*I and *Asc*I is shown **Figure 4,** along with the serogroups.

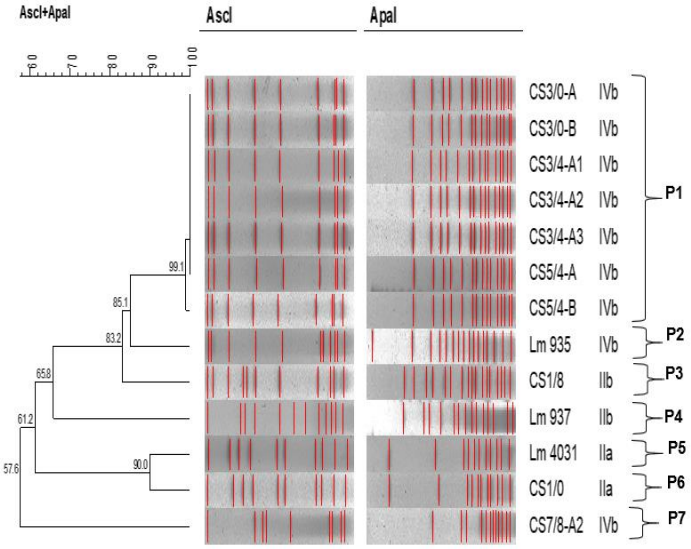

Figure 4. Dendrogram of the *Apa*I-*Asc*I profiles in PFGE and corresponding serogroups for 10 *L. monocytogenes* selected isolates, plus 3 reference isolates (*L. monocytogenes* CECT 4031, 935 and 937). P - pulsotype.

The obtained 10 *L. monocytogenes* isolates together with three reference strains (*L. monocytogenes* CECT 4031, 935 and 937) were assigned to 7 different pulsotypes (P1 to P7) with more than 95% of similarity.

Pulsotypes 2, 4 and 5 correspond to *L. monocytogenes* CECT 935 (serogroup IVb) and CECT 937 (serogroup IIb), and CECT 4031 (serogroup IIa), respectively. All the inoculated reference strains *L. monocytogenes* share less than 90% of similarity with the isolates. These results discard experimental contamination of the blank samples, pointing towards a contamination event in the producing industry.

Pulsotypes 3, 6 and 7, belonging to serogroups IIb, IIa and IVb respectively, display distinct profiles. Yu, & Jiang (2014) also found distinct profiles in approximately 30% of the studied PFGE profiles, when assessing *L. monocytogenes* isolates collected from retailed foods in Henan, China.

Pulsotype 1 includes most of the assessed isolates (70%), all belonging to serogroup IVb. These isolates were all recovered from RTE chicken salad batches 3 and 5 (collected on March 7, 2018 and April 18, 2018, respectively). These results seem to indicate that P1 isolates may represent a persistent contamination within the assessed food industry and might point out to a common source of contamination. However, as this pulsotype was only observed in two batches, it is difficult to conclude about a persistent contamination. Nevertheless, it is important to highlight that this possible persistent contamination involves *L. monocytogenes* serogroup IVb isolates, commonly associated to human disease, present in a RTE chicken salad that will not undergo any heat-treatment prior to consumption (Maury et al., 2016; Montero et al., 2015). A thorough sampling plan should be considered during a prolonged time frame, to conclude on the persistence of *L. monocytogenes* strains in the assessed food industry. For that purpose, food related environment and raw materials should also be considered in the sampling scheme.

3.6. RT-qPCR

A comparison of the obtained *Listeria monocytogenes* concentrations on the last day (8th day) of the assay, using PMA-qPCR technique and cultivation-based techniques (viable cell count on ALOA media) is shown in **Figure 5.**

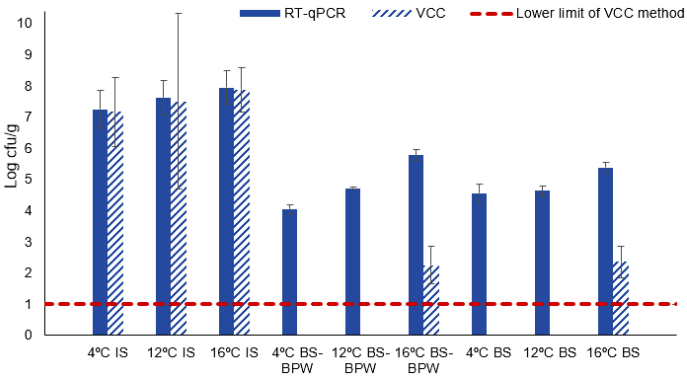

Figure 5. *L. monocytogenes* levels (log cfu/g) obtained by PMA-qPCR and VCC, on the final day of each assay. The average and SD are shown (qPCR n=6, and VCC n=3). Lower limit of VCC method is represented by the dotted line.

For every assay, the log cfu/g of *L. monocytogenes* obtained by PMA real time quantitative PCR was higher than the ones obtained by VCC in ALOA. For quantification of RTE chicken salads samples inoculated with *L. monocytogenes* (4ºC IS, 12ºC IS and 16ºC IS), no significance difference was detected between the two quantitative methods, for each assay ($p < 0.05$). However, for both quantification of RTE chicken salads blank samples (4ºC, 12ºC and 16ºC BS and BS-BPW), a significance difference was detected between the two quantitative methods, which may be attributed to the detection of VBNC bacteria only by RT-

qPCR, or due to the quantification of DNA from dead cells, showing that the PMA procedure may not have been successful.

With the results obtained by RT-qPCR coupled with PMA, it can be considered that it is a powerful approach, which allows for an easier, sensitive, specific and time-saving *L. monocytogenes* quantification, this is especially important when considering RTE foods due to their short commercial shelf-life (Agustí et al., 2018; Postollec et al., 2011). Nonetheless, due to high discrepancies when comparing the levels of *L. monocytogenes* on blank samples with the classical method (ISO 11290-2:2017), optimization and validation of the developed PMA-qPCR are essential before its application as a routine tool in microbial sampling programs, in food industry.

3.7. Modelling *L. monocytogenes* growth on artificially inoculated salads RTE chicken salads

L. monocytogenes 3-strains mix countings, as recovered from inoculated RTE chicken salads, under the considered isothermal conditions (4°C, 12°C and 16°C), are shown in, with fitted growth curves generated using the Baranyi model (1994).

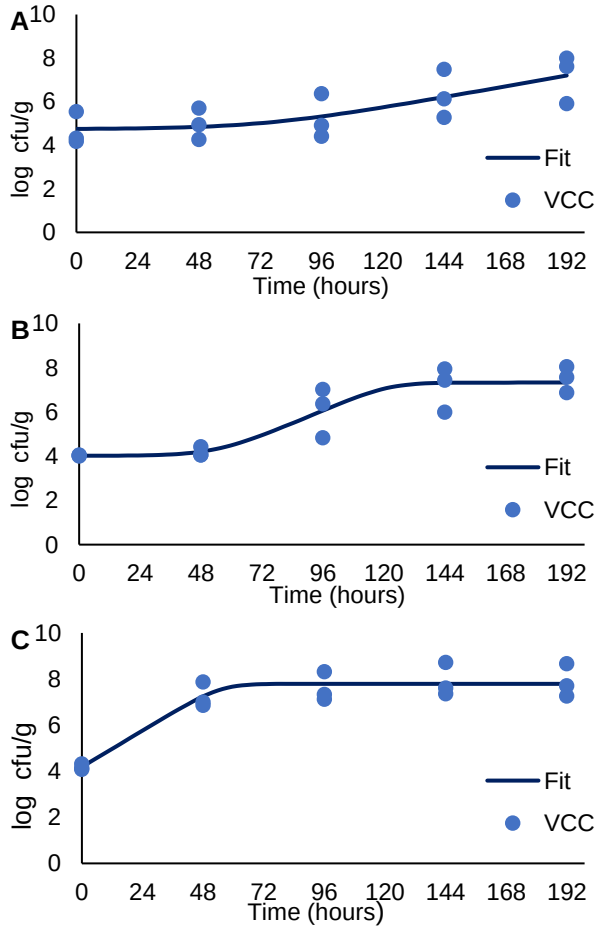

Figure 6. *L. monocytogenes* countings obtained from inoculated RTE chicken salad stored at **(A)** 4°C, **(B)** 12°C and **(C)** 16°C and growth prediction curves based on the Baranyi's model (Baranyi, & Roberts, 1994).

The growth parameters of *L. monocytogenes* on RTE chicken salads stored at different temperatures predicted by primary model described by Baranyi and Roberts are presented in **Table 3** (maximum specific growth rate (μ_{max}), lag time (λ), initial cell count (C_0), and maximum population density (N_{max}).

Table 3. Growth parameters of *L. monocytogenes* in RTE chicken salads, inoculated with the pathogen and stored at different isothermal conditions.

Growth kinetic parameter[a]	Storage temperature		
	4°C	12°C	16°C
μ_{max} (log cfu/g/h)	0.021±0.008	0.052±0.024	0.066±0.009
λ (h)	74.435±48.466	54.139±31.566	_[b]
C_0 (log cfu/g)	4.751±0.418	4.019±0.410	4.184±0.321
N_{max} (log cfu/g)	_[b]	7.325±0.300	7.792±0.185
R^2	0.512	0.807	0.870

[a] Values are means ± standard deviations (n=3).
[b] No growth kinetic parameter value was estimated.

Data obtained in the primary model of growth (values of μ_{max} (**Table 3**)) was used to elaborate a secondary model according to the square root model described by Ratkowsky et al. (1982), which allowed to predict the μ_{max} described on the basis of the temperature variation. The developed model was able to assess the growth of *Listeria monocytogenes* on RTE chicken salads under sub-optimal temperatures. **Equation 5,** describing the relationship of μ_{max} and temperature for *L. monocytogenes* grown in these RTE chicken salads under suboptimal temperatures is shown above:

$$\sqrt{\mu_{max}} = 0.0094(T + 11.745) \quad \textbf{(5)}$$

As can be observed in **Figure 6**, as storage temperature increased, the lag time (λ) decreased, with values of 74.435 ± 48.466 hours (4°C) and of 56.139 ± 31.566 hours (12°C). The lag time was not evident at 16°C. In contrast, the maximum specific growth rate (μ_{max}) increased gradually as the storage temperature increased, with values of 0.021 ± 0.008, 0.052 ± 0.024, and 0.066 ± 0.009 log cfu/g/h at 4º, 12º, and 16ºC, respectively. Significant differences ($p < 0.05$) were detected in μ_{max} for the three different temperatures, using one-way ANOVA statistical analysis, revealing that temperature has influence on the pathogen's growth in these RTE salads. However, when applying Tukey's multiple comparison test, significant differences ($p < 0.05$) were only observed between 4º and 16ºC. Similar observations under chilling conditions have been made by other authors regarding fresh vegetables and mixed salads (De Cesare et al., 2018; Omac et al., 2018; Sahu et al., 2016).

The fitting of the secondary square-root-type model to the estimated μ_{max} at each of the tested isothermal conditions resulted in the estimation of the theoretical

minimum temperature that allows microbial growth (T_{min}). The estimated values of T_{min} for RTE chicken salads was -11.745°C, with R^2=0.993. However, the European Union Reference Laboratory for *Listeria monocytogenes* (EURL Lm, 2014), indicates -1.5ºC, as minimum growth temperature for *L. monocytogenes*. Such difference may relay on the fact that this value was based on research carried out primarily in lab media under optimum conditions and may vary depending on the strain and food matrix (Li et al., 2016; Lianou et al., 2017).

3.8. Quantitative microbial risk assessment

All the information gathered in QMRA steps was integrated to provide a description of the risk of listeriosis by the ingestion of RTE chicken salad contaminated with *L. monocytogenes*. The three possible scenarios regarding the number of listeriosis cases associated with the consumption of RTE chicken salads (high risk, low-risk, and total population (combined) are shown in **Table 4**. The more realistic scenario (combination of high risk, low-risk population) of the annual number of listeriosis due to the consumption of RTE chicken salads is shown in **Figure 7** by means of a frequency distribution**.**

Table 4. Number of listeriosis cases associated with the consumption of RTE chicken salads from the studied industry (Minimum, maximum and mean (CI 95%)).

Population	Minimum	Maximum	Mean (CI 95%)
High-risk	1.900×10^{-4}	5.654×10^{-3}	1.975×10^{-3} (9.311×10^{-4}- 3.242×10^{-3})
Low-risk	4.336×10^{-5}	1.290×10^{-3}	4.509×10^{-4} (2.125×10^{-4}- 7.390×10^{-4})
Total	4.336×10^{-5}	5.654×10^{-3}	1.213×10^{-3} (2.538×10^{-4}- 2.925×10^{-3})

As shown in **Table 4**, based on Monte Carlo simulations, the average number of listeriosis cases per year linked to the consumption of these RTE chicken salads was 1.213×10^{-3} (CI 95%: 2.538×10^{-4} - 2.925×10^{-3}).

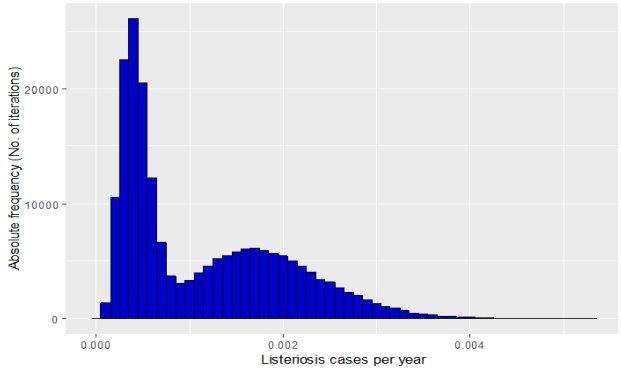

Figure 7. Frequency distribution of annual number of listeriosis cases due to the consumption of RTE chicken salads produced on the industry of the study. *Graphic obtained from ggplot2 (package of R).*

Moreover, when comparing the estimated number of listeriosis cases in low- and high-risk subpopulations (represented by both peaks in **Figure 7**), it is possible to observe that high risk population has a risk of listeriosis of four times higher. Thus, particular preventive tips should be given to high risk population.

In Portugal in 2016, a total of 32 cases of listeriosis were reported, with the highest rates detected in infants below one year of age (2.34 per 100000 population), people between 45 and 64 years of age (0.52 per 100 000 population) and elderly people over 65 years of age (0.61 per 100 000 population) (EFSA & ECDC, 2017). The average number of cases of listeriosis per year linked to the consumption of these RTE chicken salads is low when compared with the total number of cases in Portugal in 2016, which can be linked to the fact that infants and elderly people, with high notification rates are not the typical consumers of these types of salads (and by that reason were not considered high risk population in the study). Moreover, even the number of cases of listeriosis attributed to high-risk groups (especially immunocompromised patients) may be overestimated, since most of these people are often advised to avoid the consumption of these type of products, exactly because of the potential risk that they represent to health (CDC, 2018; Chau et al., 2017).

3.9. Sensitivity analysis

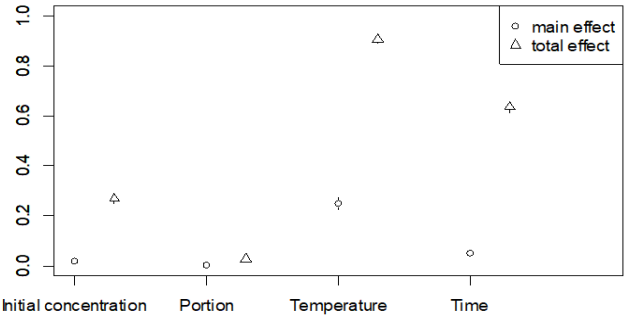

Figure 8. Sensitivity risk factors affecting the risk of listeriosis per dose, due to the consumption RTE chicken salads. *Graphic obtained from R.*

The sensitivity analysis (**Figure 8**) allowed to determine that household' storage temperature and duration of storage are the most influential factors for the risk of listeriosis derived from the consumption of RTE chicken salads. Thus, consumers should be educated and informed about good conservation practices, and provided sufficient information regarding risk issues, being this a responsibility shared by food suppliers, educators and governments. Similar results in terms of the most influential factors for the risk of listeriosis were obtained by Ding et al. (2013), Sant'Ana et al. (2014) and Tromp et al. (2010), when assessing the risk of listeriosis derived from the consumption of leafy greens intended to be eaten raw.

Initial *L. monocytogenes* concentration also had an average contribution to the risk of listeriosis, thus an intervention to be applied would be the improvement of hygienic practices during RTE chicken salads production.

4. Conclusion

The pH and a_w results confirmed this study's RTE chicken salad as a food product able to support the growth of *L. monocytogenes*. Also, the results highlighted that temperature influenced the growth of *L. monocytogenes* in these RTE chicken salads.

This work underlined the importance of strict temperature control from processing to consumption. Refrigerated temperatures must be maintained during transportation, distribution, storage or handling in supermarkets and by consumers, however, it is noteworthy to emphasize that results showed that *L. monocytogenes* will still be able to grow at low refrigeration temperatures and reach unsafe concentrations, if there is storage time abuse.

Experimental data on *L. monocytogenes*' growth at different temperatures in this study, was used to develop predictive growth models, which may be applied by the food industry and regulatory agencies to estimate the growth of *L. monocytogenes* in similar types of RTE foods

Genetic typing data indicated that some of the *L. monocytogenes* isolates detected on blank samples from serogroup IVb might represent a persistent contamination within the assessed food industry and might point out to a common source of contamination.

Due to the rare development of microbiological risk assessment in Portugal, this study may contribute to a better understanding and prediction of listeriosis cases by consumption of contaminated RTE products, and subsequently improve risk management and strengthen food control.

Acknowledgement

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Annex. Overview of the model and the parameters with their values and/or distributions, and source.

Variable	Definition	Unit	Formula/distribution	Source
P	Prevalence	%	Beta (6 + 1; 27 - 6 + 1)	Calculated from data
C_0	Initial concentration	Log cfu/g	ECDF (c (-1.4, -1, 0, 1, 2), min= -1.4, max = 3, prob = c (17, 1, 1, 2, 1)	(Carrasco et al., 2010; Gombas et al., 2003)
T	Home storage temperature	ºC	Pert (3.2ºC, 6.4ºC and 11ºC)	Obtained from household monitoring
t	Home storage time	h	Uniform (0,192)	Assumption based on shelf-life
G	Growth during home storage	Log cfu/g/h	$\sqrt{\mu_{max}} = b(T - T_{min})$	Calculated - Obtained from challenge test (Ratkowsky et al., 1982)
N_{max}	Maximum achievable viable cell count	Log cfu/g	7.8	Calculated
C_f	Concentration after storage	Log cfu/g	$C_f = \log_{10}(10^{N_{max}}) - \log_{10}\left(1 + (10^{\log_{10} 10^{N_{max}}}) - \log_{10}(10^{C_0})) - 1 \times e^{(-G \times t)}\right)$	Adapted from Baranyi & Roberts (1994) model with no lag
S	Serving size	g	Pert (82.5, 165, 247.5)	Assumption
D	Ingested dose	cfu	$D = 10^{C_f} \times S$	Calculated
Po_{risk}	Population at risk	%	8.96	Calculated
r	Probability of infection from 1 cell	-	Low risk population: 2.37×10^{-14} High risk population: 1.06×10^{-12}	(FAO/WHO, 2004)
P_{ill}	Probability of infection	-	$P_{ill}(D; r) = 1 - e^{(-r \times D)}$	(FAO/WHO, 2004)
$MP_{ill}H/L$	Mean probability of infection High or Low population	-	Mean (P_{ill})	Calculated
N_p	Number of portions consumed per year	-	344402	Annual production of the factory
N_{cont}	Contaminated portions consumed	-	$N_p \times P$	Calculated
$N_{cont}H$	Contaminated portions consumed by high-risk population	-	$N_{cont} \times \frac{Po_{risk}}{100}$	Calculated
$N_{cont}L$	Contaminated portions consumed by low-risk population	-	$N_{cont} - N_{cont}H$	Calculated
N_cH	Number cases per year in high-risk population	-	$\text{Cont Ns} \times MP_{ill}H$	Calculated
N_cL	Number cases per year low-risk population	-	$\text{Cont Ns} \times \text{MP}_{\text{ill}}\text{L}$	Calculated
N_c	Number cases per year	-	$N_cH + N_cL$	Calculated