Microbiological challenge testing for *Listeria monocytogenes* in ready-to-eat food: a practical approach

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Abstract

Food business operators (FBOs) are the primary responsible for the safety of food they place on the market. The definition and validation of the product's shelf-life is an essential part for ensuring microbiological safety of food and health of consumers. In the frame of the Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs, FBOs shall conduct shelf-life studies in order to assure that their food does not exceed the food safety criteria throughout the defined shelf-life. In particular this is required for ready-to-eat (RTE) food that supports the growth of *Listeria monocytogenes*. Among other studies, FBOs can rely on the conclusion drawn by microbiological challenge tests. A microbiological challenge test consists in the artificial contamination of a food with a pathogen microorganism and aims at simulating its behaviour during processing and distribution under the foreseen storage and handling conditions. A number of documents published by international health authorities and research institutions describes how to conduct challenge studies. The authors reviewed the existing literature and described the methodology for implementing such laboratory studies. All the main aspects for the conduction of *L. monocytogenes* microbiological challenge tests were considered, from the selection of the strains, preparation and choice of the inoculum level and method of contamination, to the experimental design and data interpretation. The objective of the present document is to provide an exhaustive and practical guideline for laboratories that want to implement *L. monocytogenes* challenge testing on RTE food.

Introduction

Regulation (EC) No 852/2004 (European Commission, 2004) on the hygiene of foodstuffs states that the primary responsibility for food safety rests with the food business operators (FBOs), which are legally responsible for the determination of the date of minimum durability of the foodstuffs they place on the market. According to Regulation (EC) No 2073/2005 (European Commission, 2005) on microbiological criteria for foodstuffs, FBOs shall ensure the compliance of their products with the limits set by the Regulation until the end of the shelf-life. When defining the product shelf-life FBOs should base their decision on scientific evidences. In particular, for ready-to-eat (RTE) food that support the growth of *Listeria monocytogenes*, the Regulation describes a series of studies that may be conducted. Among the indicated shelf-life studies are the challenge studies. Although the Regulation indicates the opportunity of conducting such studies, it does not describe how to perform them. Two main guidance documents have been published describing the methodology to conduct shelf-life studies for *L. monocytogenes* in RTE food. The first, directed to laboratories, is a technical guidance document on shelf life studies for *L. monocytogenes* in RTE food, prepared by the EU Community Reference Laboratory (CRL) for *L. monocytogenes* (Beaufort *et al*., 2014). The second document, intended for FBOs, is the Guidance document on *L. monocytogenes* shelf-life studies for RTE foods, under Regulation (EC) No. 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs (EC/DG SANCO, 2008). There are a number of existing available documents published by health authorities at international scale supporting the implementation of challenge testing as a control measure of *L. monocytogenes* in RTE foods (USFDA, 2008; Chilled Food Association, 2010; NZFSA, 2011; Health Canada, 2012; FSIS, 2014). A challenge study consists in the artificial contamination of the food with the target microorganism under controlled experimental conditions. These studies are intended to determine whether a RTE food is able to support the growth of *L. monocytogenes* or not during the designated shelf-life. A further application of challenge studies is to validate the efficacy of lethality treatment applied to RTE foods intended to reduce or eliminate the pathogen (Scott *et al.*, 2005). Many factors should be taken into account in designing, conducting and interpreting the results of a challenge study. Some of them are related to the laboratory conditions while other are related to product formulation, manufacturing process, packaging, conditions during distribution and consumption. To neglect all these aspects could lead to flawed conclusions and invalidate the study. Therefore, the aim of the present document is to review the existing available documents and to provide FBOs, research laboratories and official control authorities with a practical guide to design and perform challenge studies for *L. monocytogenes* in RTE foods.

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Materials and Methods

Laboratories performing challenge studies should be aware that for handling *L.monocytogenes* a biosafety level 2 is required and expert microbiologists are needed. Several factors must be considered when conducting a challenge study. A brief description of all the main aspects follows.

Selection of *L. monocytogenes* strains

It is usually recommended to use a pool of at least three to five different strains so that differences in growth and survival among strains are taken into account. The inoculum should include strains isolated from the processing environment or from outbreaks associated with the food being tested (Scott *et al*., 2005). The serotypes most frequently involved in human listeriosis (1/2a, 1/2b and 4b) should be part of the inoculum. Strains obtained from international culture collections, *i.e*. American Type Culture Collection (ATCC) or National Collection of Type Cultures (NCTC) can also be used. However, wild type strains are more likely to adapt and growth on the food matrix as compared to reference strains (Guyer and Jemmi, 1991; Skalina and Nikolajeva, 2010; Spanu *et al*., 2012, 2013). Alternatively to the use of pathogens, surrogate microorganism can be used (*i.e*. *Listeria innocua*) when the study is to be conducted into a processing facility. These microorganisms have similar characteristics with the target microorganism, except for the pathogenicity. Although it is reasonable to assume that surrogates have similar behaviour, they should be tested to demonstrate similar growth and resistance as com-

pared with *L. monocytogenes* and for possible interaction with food formulation and background microflora (Scott *et al*., 2005). After the selection of the strains a genetic characterization should be conducted in order to determine if the strains recovered from the challenged samples are the same that were inoculated. This characterization could be performed with several methods such as Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Pulsed Field Gel Electrophoresis (PFGE), DNA microarrays and gene sequencing.

Preparation of the inoculum

The procedure for the preparation of the inoculum should always start from strains stored at -80°C with glycerol, avoiding subculturing of strains for more than five passages (AOAC, 2006). Streak strains onto non selective agar medium (*e.g.*, trypticase soy agar, TSA or brain heart infusion agar, BHIA) and incubate for 24 h at 37°C. Pick a single pure colony and transfer into tubes containing non selective nutrient broth (trypticase soy broth, TSB or brain heart infusion broth, BHI) and incubate at 37°C for a time sufficient for the strains to reach the same physiological state (late exponential phase or early stationary phase). Overnight incubation up to 36 h, depending on the use of static or shaken incubation, is usually appropriate to obtain cells in stationary phase (*ca*. 1 x 10^9 cells/mL). Prepare a second subculture in broth medium and incubate at refrigeration temperature for the time sufficient to reach the late exponential phase or early stationary phase. Preliminary test should be performed to determine the incubation time and enumeration confirmed by colony counting on agar plates. This phase is essential to adapt the strains when the challenge is conducted in RTE refrigerated foods. This may require also adaptation to pH, water activity (a_w) or other hostile conditions (*e.g.* NaCl concentration, preservatives) characteristic of the tested food. The preparation of each of the strains to be mixed in the inoculum should be performed separately. Once adapted, mix individual cultures in equal volume to obtain a working stock solution, which will be used, after appropriate dilution, for the contamination of the product.

Inoculum level

Prepare adequate serial dilutions in phosphate buffered saline (PBS) or sterile saline solution (0.85% NaCl) to obtain the desired level of contamination.The level of the inoculum to be used depends on the objective of the study. If we were to determine the growth of *L. monocytogenes* or the stability of a product formulation, it is usually recommended to obtain a final concentration between 10^2-10^3 cfu/g of product (USFDA, 2001; Uyttendaele *et al*., 2004; Scott *et al*., 2005; Beaufort *et al*., 2014; NACMCF, 2010; Augustin *et al*., 2011). Although natural contamination of most foods in generally lower, this level allows enumeration of *L. monocytogenes*. In some circumstances lower levels can also be used but in this case the detection limit of the enumeration method should be increased, by using duplicate plates (*e.g.* 2 mL of the suspension onto 6 plates) or by using the most probable number (MPN) method (NACMCF, 2010; Corry *et al*., 2010). Failure to enumerate *L. monocytogenes* could lead to the incorrect conclusion that the product is safe. On the other hand, if too high of an inoculum level is used, the microorganisms may overcome the ability of the product formulation to inhibit *L. monocytogenes* growth. Instead, the validation of a lethal treatment requires higher inoculum levels. This depends on the extent of reduction we desire to validate. Inoculum levels of approximately 10^5-10^7 cfu/g of product are generally suggested (USFDA, 2001; Scott *et al*., 2005; NACMCF, 2010). According to the *Listeria* Rule issued by the Food Safety and Inspection Service (FSIS) the validation of a post-lethality treatment should demonstrate at least 1-log reduction of *L. monocytogenes* to be considered effective (FSIS, 2003), but higher levels of inactivation can be demonstrated according to circumstances. The Codex Alimentarius Commission (2004) defined the effect on the frequency and/or concentration in a food that must be achieved by the application of one or more control measure to provide or contribute to a food safety objective (FSO) or adequate level of protection (ALOP), as the performance criterion (PC). Different public health risk for *L. monocytogenes* are accepted by international health authorities which lead to a *zero tolerance* policy in RTE meat products recommended by USDA, while the SCVPH of EC (2005) considered 10^2 cfu/g a FSO at the time of consumption valid to provide an ALOP. When selecting the appropriate PC, FBOs should consider the type of food, the possible survival, growth and recontamination during the product shelf-life.

Experimental design

A number of factors should be taken into account when designing challenge studies. The duration of the study should be at least equal to the shelf-life of the product and analysis performed at least the day the product is inoculated (*day 0*) and at the end of the shelf-life (*day end*). A sufficient number of intermediate sampling intervals (at least 4-5) should be set over time. Ideally, an additional time should be considered (1.5 times the shelf-life), to simulate the eventuality the product is consumed beyond its assigned durability (Scott *et al*., 2005). At each interval a minimum of 3 inoculated test units should be analyzed. Increasing the number of the units tested at each analysis point will increase the confidence of the study. Along with inoculated test units, a number of control units should be analyzed at each sampling interval. Controls are represented by uninoculated units (*blank samples*) used for detecting the level of natural contamination with *L. monocytogenes*, the background microflora and physical-chemical characteristics of the product. The determination of the background microflora is essential in order to evaluate possible interaction that may affect the growth of *L. monocytogenes*. The physical-chemical properties of the product (*i.e.* a_w, moisture, salt level, pH, preservatives levels, gas concentrations in Modified Atmosphere Packaging, *etc*.) should be monitored through the shelf life to account for factors that may affect the growth or inactivation rate of *L. monocytogenes*. As far as sample size, duplicate or triplicate sample units should be tested at each interval point. The study should be ideally repeated in three independent trials using three different batches of the same product so that product variation is considered (Scott *et al*., 2005; Beaufort *et al*., 2014).

Inoculation method

The inoculation procedure should be performed in such a way that product formulation is not changed. Therefore, the inoculum volume should not exceed 1% of the product weight or volume. For products that are packaged in MAP the inoculation procedure shall ensure that the gas composition is similar to what is expected in the uninoculated products (Beaufort *et al*., 2014). Contamination should be as close as possible to natural contamination. Liquid product can be directly inoculated with an appropriate volume of the mixed culture at the desired concentration, while solid products can be sprayed, dipped, spreaded or mixed with the inoculum. A holding period after the inoculation is needed to allow the inoculum to attach to the product (Health Canada, 2012). The level of contamination should be confirmed by testing control positive unit after the inoculation.

Food product storing conditions

After the artificial contamination and for the entire duration of the study the products should be packaged under the same condition as intended for marketing (under vacuum, modified atmosphere, *etc*.). The temperature should mimic the foreseen conditions of the product during storage and distribution (*e.g.* refrigerated). Although out of the control of food industries, poor consumers handling of the product during dispatch, storage and domestic usage should be taken into account. Therefore, temperature abuse in the distribution chain could also be simulated by incubating the food at temperatures above the refrigeration temperature. The time and the temperature used should be justified by detailed infor-

mation: the 75th percentile of the observation for the country where the stage of cold chain is located (Beaufort *et al*., 2014).

Samples analysis

The detection and enumeration of *L. monocytogenes* should be conducted according to standard methods (ISO 11290-1:1996 and ISO 11290-2:1998; ISO, 1996, 1998) as stated in the Regulation No. 2073/2005. When the study is aimed to evaluate the efficacy of a lethality step, it is required to use an enrichment method in order to detect *L. monocytogenes* that may be no longer recovered with the enumeration method. Inoculated units should be analyzed at day 0, day end and at all intermediate points for the enumeration of *L. monocytogenes*. Non-inoculated units should also be analyzed at the same intervals by the detection method. When assessing the lethality of a killing steps (*e.g*. heath treatment, high pressure), detection and enumeration method should be conducted on treated units after (the same day) the delivery of the lethal treatment. The lethal treatment may not kill all *Listeria* cells that may survive in the product. Such injured cells may repair themselves and recover their ability to growth, being as dangerous as uninjured cells. However, sublethally injured cells may not be cultured on selective media due to the presence of antibiotics, organic dyes and other selective agents. Therefore, testing foods after heat treatment requires techniques that enable to detect sublethally injured cells. Although the use of non selective media on one hand allows the recovery of damaged cells, on the other hand cannot differentiate the target microorganism from background microflora. The Thin Agar Layer (TAL) method is a method which consists in the overlay of a nonselective agar medium onto agar plates containing a selective medium that combines the ability to enumerate and to differentiate heat injured cells (Kang and Fung, 1999; Wu and Fung, 2001). Determination of the relevant physical-chemical characteristic (pH, a_w, salt content, preservatives concentration) that can affect the inactivation or growth of *Listeria monocytgenes* should be evaluated over product shelf-life. To take into account the variability of product formulation, when performing challenge study the formulation that is the most permissive for *Listeria* growth should be tested. Standard methods should be used when conducting the analysis. Gas composition should be monitored in product that are MAP packed to check if their concentration is stable throughout the entire shelf-life. In Table 1 an example of experimental design with the test units, sampling point and analysis to be conducted when performing a challenge study is reported. Testing for background microflora in control units gives important indication of their effect on the shelf-life of the product. The presence of starter (*i.e*. lactic acid bacteria) can compete with *L. monocytogenes* limiting its growth, while other contaminant microorganisms can spoil the product before *L. monocytogenes* could grow to risk levels.

Results

Assessing growth potential

The growth potential (δ) is defined as the difference between the $\log_{10}$ cfu/g at the end of the test and the $\log_{10}$ cfu/g at the beginning of the test (Beaufort *et al*., 2014). The $\log_{10}$ cfu/g at day 0 and the $\log_{10}$ cfu/g at day end are obtained taking the median of the $\log_{10}$ cfu/g concentration among the test units at the beginning and at the end of the study, respectively. Their difference is computed independently for each batch. The maximum difference between these values is the growth potential. A food is considered able to support the growth of *L. monocytogenes* if the δ is higher than 0.5 $\log_{10}$ cfu/g, while it is assumed that the food is not able to support the growth if the δ is lower than 0.5 $\log_{10}$ cfu/g. In Table 2 a selection of studies assessing the growth potential of *L. monocytogens* obtained in different RTE food after artificial contamination is reported.

Assessing lethality

In this case it is not always necessary to analyze inoculated units at each sampling point. Since the objective is to validate the lethality of a process it is necessary to examine

Table 1. Experimental design indicating the type of analysis, the testing time and the relative minimum number of test units to perform per batch.

Analysis	Test units	Testing time				
		T_0	T_1	T_2	T_n	T_{end}
Detection and enumeration of *L. monocytogenes*	IU	3	3	3	3	3
	NC	3	3	3	3	3
	BS	3	-	-	-	-
Background microflora	NC	3	3	3	3	3
	BS	3	3	3	3	3
Physico-chemical characteristics	NC	3	3	3	3	3
	BS	3	3	3	3	3

IU, units inoculated with *Listeria monocytogenes*; NC, negative control, inoculated with sterile physiological water; BS, uninoculated blank samples.

Table 2. Examples of studies aimed to assess the growth potential in different ready-to-eat food artificially contaminated with *Listeria monocytogenes*.

Type of food	RTE food	pH	A_w	Incubation		Growth	Reference
				Temperature (°C)	Time (days)	($\log_{10}$ cfu/g)	
Meat products	Cooked ham slices	6.2	0.975	7	5	2.0	Uyttendaele *et al.*, 2004
	Sliced deli meat	6.1-6.3	-	7	35	7.0	Beumer *et al.*, 1996
Dairy products	*Ricotta salata cheese*	5.8-6.3	0.940-0.950	4-6	60	3.05-4.87	Spanu *et al.*, 2012
Fishery products	Smoked salmon	5.8-6.3	0.93-0.96	4-10	30	2.5-4.5	Guyer and Jemmi, 1991
	Salmon preparations	-	0.997	4-8	7	1.3-6.42	Midelet-Bourdin *et al.*, 2010
Produce and salads	RTE vegetables	6.2-7.2	-	7-15	6	0.21-3.34	Sant'Ana *et al*., 2012
	Green leafy vegetables	-	-	7-10	-	0.5-1.5	Carlin and Nguyen–The, 1994
	Garlic cheese salad	5.5	-	3-7	2	0.44-0.99	Skalina and Nikolajeva, 2010
	Smoked ham salad	5.0-5.1	-	3-7	2	0.26-1.11	Skalina and Nikolajeva, 2010
	Shrimp-tomato salad	5.5	-	3-7	2	0.48-0.64	Skalina and Nikolajeva, 2010

RTE, ready-to-eat; a_w, water activity.

the product at the start of the trial, after the process (treatment) and at the end of its designated shelf-life (end-point determination). To determine if the process is capable of deliver the required level of lethality against *L. monocytogenes* (performance standard or *D*), the difference between the level of log_{10} cfu/g after the inoculum and at the end-point is computed. As for the determination of the growth potential, the log reduction should be calculated independently for each batch. To account for a margin of safety the lowest log reduction obtained should be compared with the highest expected contamination. The result obtained can be expressed as log reduction of the target microorganism and the performance standard *D* is the number of log reduction. FSIS require for a post-lethality treatment to be validated to demonstrate at least 1-log reduction (*i.e.* a 90% reduction of the pathogen), while a reduction of 5 log is considered a full lethality treatment (FSIS, 2014). If microbiological challenge testing fails to demonstrate the predetermined level of *D*, the study is not invalid, a lower *D* can still be validated. In Table 3 a selection of studies assessing the effectiveness of thermal pasteurization, irradiation and high-pressure decontamination technologies applied on different RTE food artificially contaminated with *L. monocytogenes* is reported.

Discussion

Microbiological challenge tests are a tool aimed to simulate the behavior of pathogens or spoilage microorganisms on a food during processing and distribution under the foreseen storage and handling conditions. They consist in laboratory based study in which the food is artificially contaminated with a known initial concentration of the target microorganism. Microbiological challenge study can be used to determine whether or not a food supports the growth of pathogenic microorganism or as performance criterion of a process intended to deliver a lethal effect. It is worth conducting challenge studies on RTE food when their formulation does not guarantee to prevent the growth of the microorganism during the designated shelf-life. The increased demand of minimally processed RTE food poses a special attention in the definition of their shelf- life. In fact, these foods are generally characterized by mild heat treatment, minimal preservatives concentration in the formulation and storage at refrigeration temperatures. This may be inadequate to kill or to prevent the growth of an important pathogenic microorganism such as *L. monocytogenes* (Peck, 2006). Furthermore, the risk of listeriosis associated with RTE food is increased by the always more extended shelf-life required by the market, giving the opportunity to *L. monocytogenes* of growing to levels exceeding the limit set by health authorities. The definition of the durability of RTE foods should be based on studies aimed to assess the ability of *L. monocytogenes* to growth or to survive in the product under the foreseen storage condition for the entire shelf-life. Durability studies, assessing the growth of *L. monocytogenes* in naturally contaminated foods, can also be conducted. Although more realistic, the drawbacks of durability studies are that the interpretation of the results is complicated by the probability of testing contaminated food samples (which depends by the prevalence of the contamination), the low level and the uneven distribution of the initial contamination. As an example of

Table 3. Examples of studies aimed to assess the efficacy of different post-lethality treatment on Listeria monocytogenes counts applied in artificially contaminated ready-to-eat food.

Type of treatment	Type of product	RTE food	Parameter	Performance standard Δ° (log_{10})	Reference
Hot water bath temperature (°C)	Meat products	Sliced deli meat	65 for 10"-5'	3.0-4.8	McCormick *et al.*, 2003; Selby *et al.*, 2006; Mangalassary *et al.*, 2008
		Sliced deli meat	85 for 10"	>6.0	McCormick *et al.*, 2003
		Deli meat	90.6-96.1 for 2'-10'	2.0-4.0	Muriana *et al.*, 2002
		Cooked turkey breast	96 for 50'	7	Murphy *et al.*, 2003b
	Dairy products	*Ricotta salata* cheese	90 for 90'	6	Spanu *et al.*, 2013
Steam pasteurization temperature (°C)	Meat products	Fully cooked frankfurters	100 for 1.5"	3.0	Murphy *et al.*, 2005b, 2006
		Fully cooked bologna logs	100 for 2.5'	2.0	Murphy *et al.*, 2005a
		Fully cooked chicken leg quarters	96 for 22'	7	Murphy *et al.*, 2003a
Electron beam irradiation (kGy)	Meat products	RTE deli meat	1.0-2.5	2.0-3.0	Foong *et al.*, 2004; Cabeza *et al.*, 2007
	Fishery products	Cold-smoked salmon	1.0-1.5	2.5-3.0	Su *et al.*, 2004; Medina *et al.*, 2009
Gamma irradiation (kGy)	Meat products	Frankfurters	0.49-2.6	1.0-5.0	Sommers and Thayer, 2000; Knight *et al.*, 2007
		RTE deli meat	0.52-2.5	1.0-5.0	Zhu *et al.*, 2005; Jin *et al.*, 2009
		Cured ham	0.75-0.90	>2.0	Fu *et al.*, 1995
	Dairy products	Feta cheese	2.5	3.0	Konteles *et al.*, 2009
	Fishery products	Seafood salad	0.7	>2.0	Foley *et al.*, 2005
	Produce	Chopped romaine lettuce	0.56	2.6-2.9	Mintier and Foley, 2006
High pressure processing (MPa)	Meat products	Frankfurters	300	1.0	Lucore *et al.*, 2000
		RTE deli meat	400-450 for 10' at 12-17°C	1.1-3.4	Morales *et al.*, 2006; Marcos *et al.*, 2008
		Dry-cured ham	600 for 5' at 15°C	3.85	Hereu *et al.*, 2012
		RTE deli meat	600-700 for 3-10' at 10-31°C	3.0-3.5	Hayman *et al.*, 2004; Jofré *et al.*, 2008, 2009
	Dairy products	*Gorgonzola* cheese	400 for 90" at 20°C	2	López-Pedemonte *et al.*, 2007
		Cheese	500 for 110" at 5-20°C	5	López-Pedemonte *et al.*, 2007
			600 for 10' or 700 for 5'	2.0	Carminati *et al.*, 2004
	Fishery products	Cold-smoked salmon	450 for 10' at 12°C	3	Medina *et al.*, 2009

RTE, ready-to-eat. °Log_{10} reduction after the application of the treatment.

the relationship existing between prevalence of microbial contamination of food and the potential for recovery, the number of test units needed to detect one or more positive per lot with 95% confidence level is 4 when the prevalence of contamination is 100%, while it increases to 299 when the prevalence is 1% (Midura and Bryant, 2001). On the other hand, challenge studies allow knowing the initial contamination level and need lower samples units to draw conclusions. However, the results are valid only for food and for the particular conditions tested. If any significant change occurs in the product formulation or in the process, the study should be repeated.

Conclusions

Challenge studies needs the support of an expert food microbiologist and should not be performed in the microbiology laboratory of the food processing plant. A well designed challenge study can be of great support for FBOs in validating lethal treatments or product formulation aimed to control survival or growth of *L. monocytogenes* for the entire shelf-life. A proper definition of the fate of *L. monocytogenes* through the processing, distribution and successive handling of RTE food is essential in order to comply with all applicable legislative and regulatory requirements. When validating the effect of an antimicrobial agent or process it should be pointed that these strategies are aimed control *L. monocytogenes* contamination in post-lethality exposed products (*e.g.* slicing, curing, packaging and other unit operations conducted after the lethality step). Therefore they should not be considered as an alternative to the implementation of proper sanitation and preventive hygienic measures.

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