



AGENCE FRANÇAISE
DE SÉCURITÉ SANITAIRE
DES ALIMENTS



EU COMMUNITY REFERENCE LABORATORY FOR
LISTERIA MONOCYTOGENES

WORKING DOCUMENT

Version 2 – November 2008

TECHNICAL GUIDANCE DOCUMENT

On shelf-life studies for *Listeria monocytogenes* in ready-to-eat foods

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GLOSSARY

Abuse temperature: temperature higher than the prescribed temperature of processing and retail storage according to national legislated temperature rules or European food regulations, including reasonably foreseeable domestic storage conditions. The abuse temperature covers the whole cold chain, taking into account in particular the temperature deviation of retail refrigerators as well as domestic storage.

Batch: group or set of identifiable products obtained from a given process under practically identical circumstances and produced in a given place within one defined production period. (Regulation (EC) No. 2073/2005).

Cold chain: the continuous system that provides chilled storage of perishable foodstuffs, from production to consumption.

Population: the group or set of units which is the field of investigation related to a given process and recipe. In practice: the studied batch.

Sampling: procedure used to draw or selection of one or more unit(s).

Sampled sub-population: the selected unit(s), supposed to be representative of the population.

Shelf-life: the period of time before the 'use by' or 'best before' date, as defined respectively in articles 9 and 10 of Directive 2000/13/EC.

Test unit: aliquot of a commercial unit, designed to be analysed.

1. Introduction

1.1. Foreword

This document was prepared by the EU Community Reference Laboratory (CRL) for *Listeria monocytogenes* in collaboration with a working group consisting of 10 laboratories, including 9 National Reference Laboratories (NRL's) for *Listeria monocytogenes*:

- National Reference Laboratory for Food Microbiology, University of Liège, Belgium,
- Department of Agriculture & Food Laboratories, (Dairy Science Laboratory), Co Kildare, Ireland,
- Laboratory of the Food and Consumer Product Safety Authority (VWA), Zutphen, The Netherlands,
- National Veterinary Institute, Oslo, Norway,
- National Food Administration, Livsmedelsverket (SLV), Uppsala, Sweden,
- National Institute of Hygiene, Department of Food and Consumer Articles Research (PZH), Warsaw, Poland,
- Institute of Hygiene and Public Health (IISPV), Bucharest, Romania,
- State Veterinary and Food Institute Dolný Kubín (SVPUDK), Slovakia,
- Experimental Institute of Animal Health (IZT) of Abruzzo and Molise, Teramo, Italy,

and:

- Campden & Chorleywood Food Research Association, Gloucestershire, United Kingdom.

The representatives of the Belgian, Dutch, Irish, Norway, Romania and Swedish NRL's and of the United Kingdom laboratory provided an active contribution.

This technical guidance document was prepared at the request of EC/DG SANCO in response to the needs expressed by EU Member States. EC/DG SANCO acknowledged that a document was required, providing both detailed and practical information on how to conduct shelf-life studies for *Listeria monocytogenes* in ready-to-eat (RTE) foods to ensure conformance to the microbiological criteria set out in Regulation (EC) No. 2073/2005.

Other documents, such as from AFNOR (2004, 2007) and Université de Liège (2007), provide national references relating to the same subject.

Annex I of Regulation (EC) No. 2073/2005 on microbiological criteria for foodstuffs sets out the criteria for *L. monocytogenes* in RTE foods. Annex II of the regulation specifies that food business operators (FBO's) shall conduct, as necessary, studies to evaluate the growth of *L. monocytogenes* that may be present in the product during the shelf-life under reasonably foreseeable storage conditions. But Annex II does not describe the procedure to conduct such shelf-life studies.

1.2. Scope

This technical guidance document is basically intended for laboratories conducting shelf-life studies for *L. monocytogenes* in RTE foods, in collaboration with the FBO's. NRL's are in charge of assisting the laboratories in their respective countries to conduct these studies. In certain cases, NRL's may conduct themselves these studies.

The present document provides recommendations on how to select, implement and perform the tests required. Another document from EC/DG SANCO, entitled "Guidance document on *Listeria monocytogenes* shelf-life studies for ready-to-eat foods, under Regulation (EC) No. 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs", is directed at FBO's who produce ready-to-eat foods.

The present document describes the microbiological procedures for determining growth of *L. monocytogenes* using **challenge tests** and **durability studies** in the frame of the application of the Regulation (EC) No. 2073/2005.

The microbiological procedures described are:

- challenge tests
 - assessing a growth potential (δ)
 - assessing the maximum growth rate (μ_{\max})
- durability studies

Use of information provided by product characteristics, scientific literature and historical data is detailed in the guidance document intended for the FBO's,

Challenge tests

Challenge tests aim to provide information on the behaviour of *L. monocytogenes* that have been artificially inoculated into a food or foodstuffs, under given storage conditions. They may take into account the variability of the foodstuffs (by using different batches) and the specific contamination of the food (by inoculating strains isolated from the food). However, the level of contamination, the heterogeneity of the contamination and physiological state of the bacteria are difficult to mimic in a challenge test study.

Challenge tests can be performed with two different objectives: either (1) assessment of the growth potential (i.e. the ability of *L. monocytogenes* to grow in the food), or (2) estimation of the growth parameters (e.g. maximum growth rate).

Challenge tests assessing growth potential

A microbiological challenge test assessing a growth potential (δ) is a laboratory-based study that measures the growth of *L. monocytogenes* in artificially contaminated food stored under foreseeable conditions of transportation, distribution and storage. A microbiological challenge test must reflect

conditions that might realistically be expected to occur throughout the cold chain, including storage conditions after production until consumption.

The growth potential (δ) is 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. The experimental results relating to δ may show a wide dispersion, notably because the lag phase is included.

δ depends on many factors, the most important being:

- the inoculated strain(s),
- the physiological state of the inoculated strain(s),
- intrinsic properties of the food (e.g. pH, NaCl content, a_w , nutritional content, associated microflora, antimicrobial constituents),
- extrinsic properties (e.g. time-temperature profile, gas atmosphere).

Among these factors, temperature may be expected to have the greatest influence on the growth of *L. monocytogenes* in a given food type.

In the frame of the application of the Regulation (EC) No. 2073/2005, δ can be used:

- to classify a food:
 - when $\delta > 0.5 \log_{10}$ cfu/g, the food is classified into "Ready-to-eat foods able to support the growth of *L. monocytogenes* other than those intended for infants and for special medical purposes" (category 1.2),
 - when $\delta \leq 0.5 \log_{10}$ cfu/g, the food is classified into "Ready-to-eat foods unable to support the growth of *L. monocytogenes* other than those intended for infants and for special medical purposes" (category 1.3),
- to quantify the behaviour of *L. monocytogenes* in a food of category 1.2 according to defined reasonably foreseeable conditions between production and consumption .
- to permit calculation of a concentration of *L. monocytogenes* at point of production, that should not lead to the level of 100 cfu/g being exceeded at the end of shelf-life.

The main advantages of this method are: (i) that it is relatively simple to implement and (ii) that results can be directly used (see above). Its drawback is the lack of flexibility in the interpretation: the results are only valid for the studied food along the studied conditions, so that new experiments have to be performed each time there is a change (e.g. the receipt is changed, different time-temperature profiles are used, ...). Also, the growth potential covers a generally long period of time (e.g. the whole shelf-life) and then cannot be used to predict growth during a limited part period of time.

Challenge tests assessing the maximum growth rate

The drawbacks of the previous approach can be solved by combining predictive microbiology models and challenge tests assessing μ_{\max} (growth rates). These experiments are more expensive and time-

consuming than the challenge tests assessing growth potential. They are restricted to cases in which predictive microbiology will be applied, by laboratories trained in predictive microbiology.

A microbiological challenge test assessing the maximum growth rate is a laboratory-based study that measures the rate of growth of *L. monocytogenes* in an artificially contaminated food stored at an appropriate temperature. The temperature used for the experiment is not (necessarily) the one used for predictions since it is possible to predict growth at another temperature than the one tested, or along a time-temperature profile chosen to be representative of the foreseeable conditions of transportation, distribution and storage.

Once the test has been performed, the maximum growth rate (μ_{\max}) of the studied *L. monocytogenes* strain at the studied temperature is calculated from the growth curve. In the exponential growth phase, plotting the natural logarithm of cell number against time produces a straight line. The slope of this line is the μ_{\max} . It is expressed, for our purpose, in day^{-1} . The maximum growth rate is an important parameter of the growth curve which depends on:

- the inoculated strain(s),
- intrinsic properties of the food (e.g. pH, NaCl content, a_w , nutritional content, associated microflora, antimicrobial constituents),
- extrinsic properties (e.g. temperature, gas atmosphere).

Then, using for example an equation suggested in the present document, it is possible to extrapolate this μ_{\max} at a temperature to predict other μ_{\max} -values at other temperatures in the same food.

Such microbiological challenge tests allow:

- an estimation of the concentration of *L. monocytogenes* at a given day of the shelf-life if the initial concentration is known,
- an estimation of the maximum allowable concentration of *L. monocytogenes* in a food that may be present on the day of production, in order to comply with the limit of 100 cfu/g at the end of shelf-life.

Durability studies

Durability studies allow an evaluation of the growth of *L. monocytogenes* in a naturally contaminated food during its storage according to reasonably foreseeable conditions.

Durability studies may be considered more realistic than a challenge test for individual foods, as the contamination is naturally occurring.

The interpretation of the results of durability studies can be difficult because of the low probability of testing a contaminated unit, the very low number of *L. monocytogenes* initially present and the heterogeneity of the distribution in the food. In these situations it may be necessary to use challenge tests to collect the information necessary to establish shelf-life and ensure compliance of ≤ 100 cfu/g at the end of the shelf-life of the product.

Durability studies can be used when *L. monocytogenes* is routinely detected in the tested food at the end of manufacturing.

Selection of appropriate microbiological procedures

The choice of the tests to be implemented should be done by the FBO, with the collaboration of the laboratory who will conduct them. The choice should be led by the information to be obtained, as illustrated in Figure 1.

Some basic rules are suggested below for this choice:

- Challenge tests for assessing δ are likely to be the "first intention" tests in most cases, especially to differentiate between products able or not to support growth of *L. monocytogenes*.
- Implementing challenge tests for assessing μ_{\max} should mostly be regarded as "second intention" tests, in specific cases in which it is expected that the additional information that they could provide may be useful. Basic knowledge about predictive microbiology is necessary to interpret results.
- Durability studies are particularly appropriate when the prevalence of *L. monocytogenes* is high.

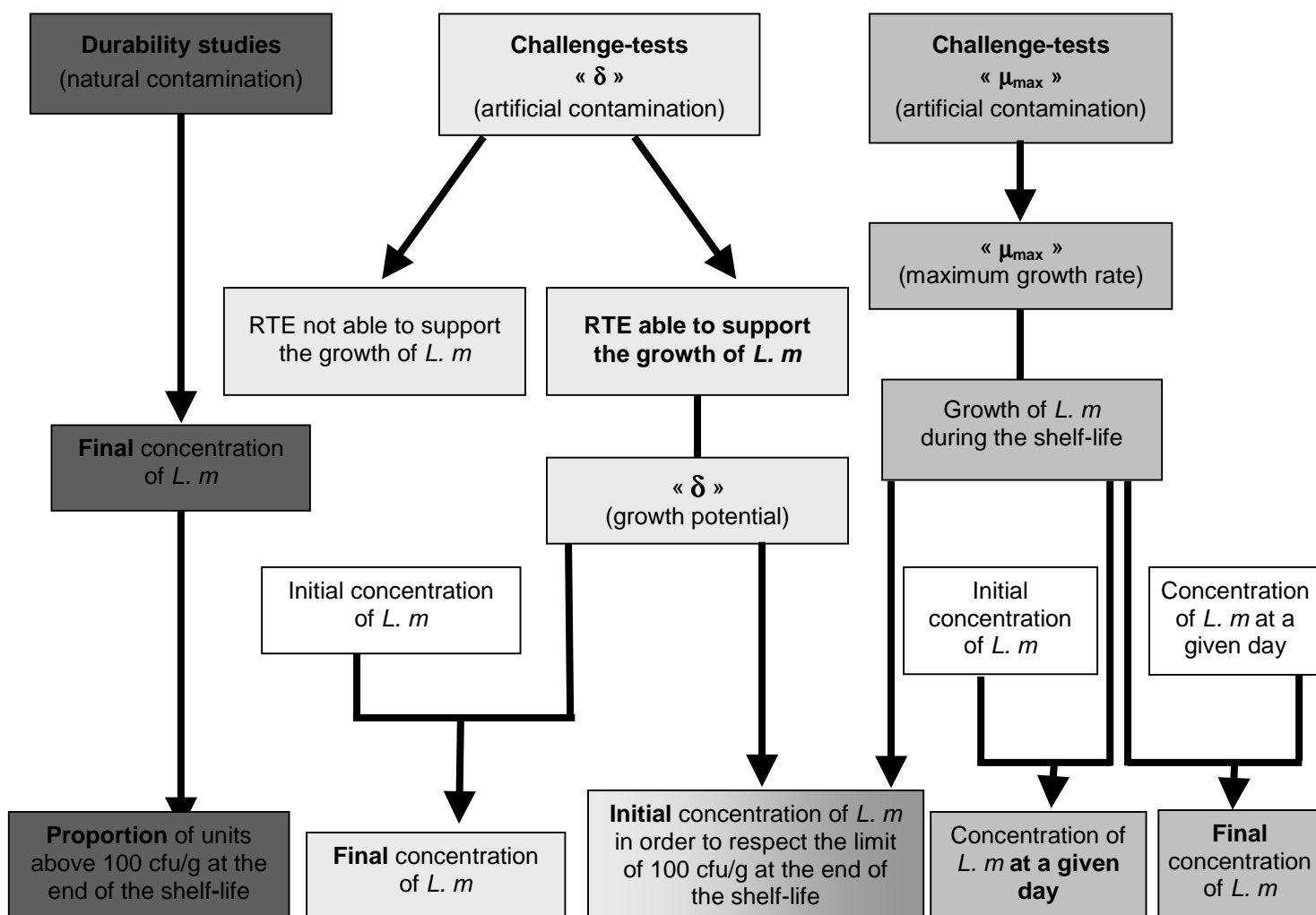


Figure 1. Data obtained from challenge tests and durability studies

2. Challenge tests assessing a growth potential

To conduct challenge tests to assess the growth potential, the following factors, as a minimum, must be taken into consideration:

- product characteristics,
- shelf-life of the product,
- number of batches,
- choice of the strain(s),
- preparation of the inoculum,
- preparation and inoculation of the test units,
- storage conditions,
- measurement of physical-chemical characteristics,

- microbiological analyses,
- calculation of the growth potential.

2.1. Product characteristics

Product characteristics at the end of the production must be described and must be representative of the variability of the characteristics of the food. These characteristics should include both intrinsic and extrinsic properties:

- physical-chemical characteristics such as pH, a_w , salt content, preservative concentration;
- associated microflora (total count) or specific microflora (e.g. lactic acid bacteria, *Pseudomonas*, ...)
- packaging conditions (air, vacuum packaging, modified atmosphere packaging).

2.2. Protocol of a challenge test to assess growth potential

2.2.1. Number of batches

Test at least 3 different batches of the same product.

2.2.2. Choice of strains

Perform microbial challenge tests with a mixture of at least 3 strains to account for variations in growth among the strains. Among the selected strains one must be a reference strain (ATCC, NCTC, CIP or equivalent). The other strains must be isolated from the same or a similar food matrix.

2.2.3. Preparation of the inoculum

Before the implementation of the challenge test, conduct prior trials to determine the time necessary to reach the stationary phase.

Subculture each strain in a medium (e.g. Tryptone Soy Broth (TSB) or Brain Heart Infusion (BHI)) and at a temperature (37°C) favourable to optimal growth of *Listeria monocytogenes*, for a sufficient time for the organism to reach the beginning of the stationary phase. This first subculture is mainly aimed at getting the cells in the same physiological state.

Prepare a second subculture and incubate it at a temperature close to the temperature of the product, in order to adapt the strain to the storage condition of the product. Incubate this culture for a sufficient time for growth of the strains to late exponential phase or early stationary phase. Combine in equal quantity the cultures from each of the 3 strains at the same concentration. Prepare successive dilutions of the mixed culture in physiological water to obtain a concentration in the foodstuff similar to one that might be realistically expected to occur naturally in the foodstuff.

Check the inoculum level on Tryptone Soy Agar (TSA).

2.2.4. Preparation and inoculation of the test units

Prepare at least the number of test units shown in table 1.

The whole experiment requires destructive sampling for microbiological procedures.

Table 1. Minimum number of test units to be prepared per batch

	"day 0" ^{a)}	"day end" ^{b)}
Determination of the concentration of <i>L. monocytogenes</i>	3	3
Detection and/or enumeration of <i>L. monocytogenes</i> in non-inoculated test units (optional)	3	3
Determination of physical-chemical characteristics	3 ^{c)}	3 ^{c)}
Determination of the concentration of the microflora	3	3

a) "day 0": the time immediately after inoculation of the product

b) "day end": the end of the shelf-life

c) one unit is enough if the FBO can demonstrate that products are homogeneous

In this table, only "day 0" and "day end" are considered, but it is highly recommended to add intermediate analysis points.

2.2.4.1. Preparation of non-inoculated test units

Test units may be used to detect and/or enumerate *L. monocytogenes* occurring naturally in the foodstuff, these "blank samples" are not inoculated. Even if *L. monocytogenes* is present in the "blank samples", the result of the challenge test is valid. It provides the additional information that naturally occurring *L. monocytogenes* strain(s) at realistic levels were present in addition to the added mixture of strains.

For determining the physical-chemical characteristics and the concentration of the microflora, do not inoculate the test units with *L. monocytogenes* but inject instead sterile physiological water.

The determination of the physical-chemical characteristics and associated microflora are necessary in order to compare the products submitted to challenge testing to the products routinely produced by the factory (see data required in § 2.1.). Moreover, the determination of the concentration of the associated microflora can bring some information about possible interactions between *L. monocytogenes* and associated microflora. Such interactions may influence the growth of *L. monocytogenes*.

2.2.4.2. Preparation and inoculation of test units for determining concentration of *L. monocytogenes*

Food

The challenge test may be performed on either a part or the whole of the commercial unit of the foodstuff. If the food is composed of several parts, the part which will be most likely contaminated with *L. monocytogenes* (e.g. the filling of a sandwich) must be the artificially contaminated. The distribution of the inoculum in the food shall mimic the plausible distribution of *L. monocytogenes* in the foodstuff, which may or may not be uniform.

Inoculation procedure

The inoculation must be as effective as possible at simulating natural contamination conditions and maintaining the intrinsic properties of the foodstuff. In order to minimise changes to the physical-chemical properties, the inoculum should not exceed 1% of the volume of the test unit, otherwise it can seriously effect the intrinsic properties of the product and thus the growth characteristics of the inoculum.

Ensure that the method of inoculation does not change the gaseous composition within the food pack, and that the gas composition within the inoculated pack during incubation is identical to the composition that would be expected to be found in a similar uninoculated pack. This may be achieved by inoculating through a cover or septum which immediately seals after the inoculating device is removed, thus maintaining correct gas condition, or by unpacking the commercial unit, inoculating the product, then repacking in a way that ensures that the gas condition is identical to that in an unopened pack.

Inoculate the foodstuff or the specific part suspected to be contaminated in a manner to mimic as closely as possible the expected natural contamination. This can be done as follows:

- in depth: for food considered to be homogeneous (e.g. ground foodstuffs) or prepared by mixing several materials (e.g. mixed salad),
- or at the surface: to mimic contamination of a specific part during process (e.g. smoked salmon contaminated during slicing).

Contamination level

Target the contamination level at 50 cfu/g, which should not exceed 100 cfu/g.

2.2.5. Storage conditions for the inoculated foodstuff

2.2.5.1. Introduction

The storage (incubation) conditions applied during challenge testing must comply with the conditions at which the product is most likely to be subjected in normal use, until its final consumption. This should include the typical temperature range at which the product is to be transported, distributed and stored.

This is a critical part of any challenge test. It is the responsibility of the FBO and laboratory to work together to ensure that the storage (incubation) conditions used are realistic, and understanding the fact that, proper storage temperatures are not always maintained throughout the cold chain (production to consumption). Therefore, challenge tests must consider the use of abuse temperature(s) as well.

2.2.5.2. Storage temperature and duration

Depending on the information available in the cold chain on storage temperatures during processing, distribution and retail, select the following storage conditions (see Table 2).

Table 2. Flow diagram of storage (incubation) conditions

Stage of cold chain	Storage (incubation) temperature			Storage (incubation) duration			
				Shelf life ≤ 21 days		Shelf life > 21 days	
From the manufacture until the arrival to the display cabinet	Temperature justified by detailed information*	Or if not known	8°C	Duration justified by detailed information	Or if not known	One third of the total shelf life of the product	7 days
Retail: Display cabinet	Temperature justified by detailed information*	Or if not known	12°C	Duration justified by detailed information	Or if not known	One third of the total shelf life of the product	½ (shelf life – 7 days)
Consumer storage	Temperature justified by detailed information*	Or if not known	12°C	Duration justified by detailed information	Or if not known	One third of the total shelf life of the product	½ (shelf life – 7 days)

* Temperature justified by detailed information: the 75th percentile of the observations for the country where the stage of the cold chain is located.

2.2.6. Measurement of physical-chemical characteristics

Measure the physical-chemical characteristics (at least pH; [NaCl content, humidity] or a_w) according to the standard methods.

2.2.7. Microbiological analyses

2.2.7.1. Detection methods

According to Annex I of Regulation No. 2073/2005, the reference detection method for *L. monocytogenes* is the standard method EN ISO 11290-1, amended. According to Article 5 of the same regulation, the use of alternative analytical methods is acceptable when the methods are validated against the reference method and if a proprietary method, certified by a third party in accordance with the protocol set out in EN/ISO standard 16140 or other internationally accepted similar protocols, is used. Other methods shall be validated according to internationally accepted protocols and their use authorised by the competent authority.

2.2.7.2. Enumeration methods

According to Annex I of Regulation No. 2073/2005, the reference enumeration method for *L. monocytogenes* is the standard method EN ISO 11290-2, amended. According to Article 5 of the same regulation, the use of alternative analytical methods is acceptable when the methods are validated against the reference method and if a proprietary method, certified by a third party in accordance with the protocol set out in EN/ISO standard 16140 or other internationally accepted similar protocols, is used. Other methods shall be validated according to internationally accepted protocols and their use authorised by the competent authority.

Since the targeted contamination level is 50 cfu/g (see § 2.2.4.2.), the limit of enumeration should be low, not higher than 10 cfu/g, thus, according to EN ISO 11290-2, using 1 ml of the initial suspension spread onto 3 plates of Ø 90 mm, or spread onto 1 large plate of Ø140 mm, or, if validated, pour-plated into 1 plate of Ø 90 mm.

In order to get a lower measurement uncertainty¹, it is highly recommended to choose a method that allows to reach an even lower limit of enumeration, e.g. 5 cfu/g, with 2 ml of the initial suspension spread onto 6 plates of Ø 90 mm, or spread onto 2 large plates of Ø 140 mm, or, if validated, pour-plated into 2 plates of Ø 90 mm. Plating 1 ml of the 10⁻² dilution (or 0.1 ml of the initial suspension) is probably unnecessary at "day 0".

A low limit of enumeration can also be obtained using filtration, or another method, if validated.

In derogation to EN ISO 7218:2007, results based on less than 4 counted colonies are expressed quantitatively, (e.g. the enumeration result associated to 3 counted colonies after plating 2 ml of the initial suspension is "1.5 cfu/g"), but they should not occur frequently at "day 0" (given a targeted contamination level of 50 cfu/g and a limit of enumeration below 10 cfu/g). For the same reasons, results below the threshold (0 counted colony) should be rare at "day 0". If they are a majority (i.e. 2 or 3 results out of 3), the challenge test (for this batch) is not acceptable.

Once each batch has been inoculated and enumerated at "day 0", it is recommended to calculate immediately the standard deviation between the 3 log-results at "day 0" (or between the 2 log-results if one of them is below the limit). If this standard deviation (due to measurement uncertainty and contamination heterogeneity) equals or is higher than 0.3 log cfu/g, then the challenge test is not acceptable.

When a challenge test is not acceptable, perform once again the experiment for this batch (or a similar one), paying great attention to the homogeneity of the contamination and to the precision of the enumeration method (increase the number of plates if necessary).

¹ This close link between measurement uncertainty and the number of counted colonies may be illustrated by the use of ISO TS 19036:2006 and its amendment.

The associated microflora taken into account may be a mesophilic aerobic count or a specific microflora of the food (e.g. lactic acid bacteria, *Pseudomonas*). Methods used to enumerate them should follow relevant CEN, ISO or national standards for the organism and food type concerned.

2.2.8. Calculation of the growth potential

The growth potential is the difference between the \log_{10} cfu/g after growth and the \log_{10} cfu/g of the initial concentration.

For each batch, calculate the difference between the median of the \log_{10} concentrations at "day end" and the median of the \log_{10} concentrations at "day 0" (\log_{10} cfu/g) . Note that the median is the intermediate result among three. If one of three results is expressed as "< limit of enumeration", it does not prevent from calculating a median, which is then the lowest of the two other results).

Then choose as δ the maximal difference between "day end" and "day 0" among the 3 batches

Examples

An example of results is shown in Table 3. In this example, it is assumed that enumeration results were obtained:

- at "day 0" by plating 2 ml of the initial suspension (10^{-1}) (either spread onto 6 plates of \varnothing 90 mm, or spread onto 2 large plates of \varnothing 140 mm, or pour-plated into 2 plates of \varnothing 90 mm), so that the limit of enumeration is 5 cfu/g,
- at "day end" by plating 2 ml of the initial suspension (either spread onto 6 plates of \varnothing 90 mm, or spread onto 2 large plates of \varnothing 140 mm, or pour-plated into 2 plates of \varnothing 90 mm) and 0.1 ml of the 10^{-1} initial suspension onto one plate of \varnothing 90 mm (or 1 ml of the 10^{-2} dilution into one plate of \varnothing 90 mm).

Table 3. First example of results obtained from a growth potential test.

The limit of enumeration is 5 cfu/g.

Batches	Day	Concentration (cfu/g)	Concentration (log ₁₀ cfu/g) In bold: median	Difference between the median concentration at "day end" and the median concentration at "day 0" (log ₁₀ cfu/g)	Growth potential (δ) = maximum of the differences
1	"day 0"	30	1.48	1.46-1.48=-0.02	0.42
		50	1.70		
		20	1.30		
	"day end"	43	1.63		
		24	1.38		
		29	1.46		
2	"day 0"	45	1.65	1.46-1.48=-0.02	0.42
		30	1.48		
		30	1.48		
	"day end"	29	1.46		
		43	1.63		
		14	1.15		
3	"day 0"	<5	<0.70	1.72-1.30=0.42	0.42
		25	1.40		
		20	1.30		
	"day end"	52	1.72		
		38	1.58		
		81	1.91		

In this first example, the standard deviations between the 3 results at "day 0" are 0.20 log₁₀ cfu/g for batch 1; 0.10 log₁₀ cfu/g for batch 2, and 0.07 log₁₀ cfu/g for batch 3, not taking into account the result below the threshold). Then, all results can be used. The maximal difference between the median concentration at "day end" and the median concentration at "day 0" (log₁₀ cfu/g) of each batch is:

$$\delta = 0.42 \text{ (log}_{10} \text{ cfu/g)}.$$

A second example of results is shown in Table 4, with the same hypothesis (threshold at 5 cfu/g).

Table 4. Second example of results obtained from a growth potential test.

The limit of enumeration is 5 cfu/g.

Batches	Day	Concentration (cfu/g)	Concentration (log ₁₀ cfu/g) In bold: median	Difference between the median concentration at "day end" and the median concentration at "day 0" (log ₁₀ cfu/g)	Growth potential (δ) = maximum of the differences
1	"day 0"	25	1.40	2.28-1.40 = 0.88	0.88
		20	1.30		
		55	1.74		
	"day end"	100	2.00		
	210	2.33			
	190	2.28			
2	"day 0"	60	1.78	2.54-1.70 = 0.84	
		30	1.48		
		50	1.70		
	"day end"	250	2.40		
		350	2.54		
	390	2.59			
3	"day 0"	20	1.30	1.72-1.30 = 0.42	
		25	1.40		
		20	1.30		
	"day end"	43	1.63		
		52	1.72		
		76	1.88		

The standard deviations between the 3 results at "day 0" are 0.23 log₁₀ cfu/g for batch 1; 0.16 log₁₀ cfu/g for batch 2, and 0.06 log₁₀ cfu/g for batch 3. Then, all results can be used. In this second example, the maximal difference between the median concentration at "day end" and the median concentration at "day 0" (log₁₀ cfu/g) of each batch is:

$$\delta = 0.88 \text{ (log}_{10} \text{ cfu/g)}.$$

2.2.9. Exploitation of the results

2.2.9.1. Ability to support growth of *L. monocytogenes*

The first question to sort out is whether the food is or not able to support the growth of *L. monocytogenes*.

If δ equals or is lower than 0.5 log₁₀, then it is assumed that the food is not able to support the growth of *L. monocytogenes*.

If δ is higher than 0.5 log₁₀, then it is assumed that the food is able to support the growth of *L. monocytogenes*.

2.2.9.2. Use of the growth potential value

In the cases in which it is assumed that the food is able to support the growth of *L. monocytogenes*, the δ value (higher than 0.5 log₁₀) may be used for predictions of growth (see example), such as:

$$\text{final concentration} = \text{initial concentration} + \delta$$

or:

$$\text{initial concentration} = \text{final concentration} - \delta$$

In practice, the final concentration obtained from the first calculation may be used to determine for a given product, with a known concentration at "day 0" whether its predicted concentration at "day end" exceeds the limit of 100 cfu/g or not.

Similarly, the initial concentration obtained from the second calculation may be used to determine a limit at the end of the production, low enough to guarantee that the limit of 100 cfu/g is not exceeded at the end of the shelf-life.

2.2.9.3. Examples

In the first example of § 2.2.8 (Table 3):

$$\delta = 0.42 \text{ (log}_{10} \text{ cfu/g)}$$

δ is below 0.5, then it is assumed that the food does not support growth of *L. monocytogenes*.

In the second example of § 2.2.8 (Table 4):

$$\delta = 0.88 \text{ (log}_{10} \text{ cfu/g)}$$

δ is higher than 0.5, then it is assumed that the food supports growth of *L. monocytogenes*. This δ -value can be used for further calculations.

If the initial concentration of *L. monocytogenes* is 1 log₁₀ cfu/g:

- What will be the concentration at the end of the shelf-life? (the initial concentration is the maximum concentration of *L. monocytogenes* in products processed according to GHP or HACCP principles at the beginning of the shelf-life).

Then, the predicted concentration of *L. monocytogenes* at the end of the shelf-life is:

$$\begin{aligned} \text{final concentration} &= \text{initial concentration} + \delta \\ &= 1 + 0.88 = 1.88 \text{ log}_{10} \text{ cfu/g} (= 76 \text{ cfu/g} < 100 \text{ cfu/g}) \end{aligned}$$

- What is the concentration of *L. monocytogenes* at the beginning of the shelf-life in order to respect the limit of 100 cfu/g? Then, a back-calculation is possible:

$$\begin{aligned}\text{initial concentration} &= \text{final concentration} - \delta \\ &= 2 - 0.88 = 1.12 \log_{10} \text{ cfu/g} (= 13 \text{ cfu/g})\end{aligned}$$

2.2.10. Test report

Include in the test report at least the following information:

- ◇ Report number,
- ◇ Information concerning full identification of the foodstuff:
 - Identification of the batches tested and their manufacturing date,
 - The characteristics of the foodstuff (pH, a_w , associated microflora, ...),
 - The intended shelf-life of the product,
- ◇ Justification of storage conditions (duration and temperature),
- ◇ Data relative to the strains under consideration:
 - Origin of the strains,
 - Inoculum preparation conditions,
 - Inoculum concentration,
- ◇ Data relative to the actual challenge test:
 - Batch numbers per product,
 - Number of test units,
 - Day of inoculation,
 - Mass or volume of the test units inoculated,
 - Inoculum volume and contamination method,
 - Storage conditions (time / temperature) of the test units,
 - Reference of the microbiological methods (enumeration and detection),
 - Limit of the enumeration method,
 - Physical-chemical characteristics of the foodstuff at the beginning and the end of the test,
 - The level of associated microflora at the beginning and at the end of the test,
 - Raw data and calculations,
 - Growth potential and interpretation.

3. Challenge test performed to assess maximum growth rate

As mentioned in the scope, this experiment is limited to specific cases, when it is expected that the specific information provided by this more complete test may be useful.

3.1. Product characteristics

See § 2.1.

3.2. Protocol of a challenge test to assess maximum growth rate

3.2.1. Number of batches

See § 2.2.1.

3.2.2. Choice of strain(s)

Test each batch with 2 strains, separately.

Before challenge testing, use growth curves in broth to select the 2 fastest strains, among isolates from the same or a similar food matrix and a reference strain (ATCC, NCTC, CIP or equivalent).

3.2.3. Preparation of the inoculum

In this case, the initial concentration of *L. monocytogenes* may be higher than the concentration expected in the product which is generally low.

Before the implementation of the challenge test, conduct prior trials to determine the time necessary to reach the stationary phase.

Subculture the strain in a medium (e.g. Tryptone Soy Broth (TSB) or Brain Heart Infusion (BHI)) and at a temperature (37°C) favourable to optimal growth of *Listeria monocytogenes* for a sufficient time to reach the beginning of the stationary phase.

Perform a second subculture under identical conditions.

3.2.4. Preparation and inoculation of the test units

Prepare at least the number of test units shown in table 5.

Table 5. Number of test units to be prepared per curve

	Test units
Determination of μ_{\max} (growth curve)	10 to 15
Detection of <i>L. monocytogenes</i> in the foodstuff prior to the challenge test ("day 0") and enumeration at the end ("day end")	3 + 3
Determination of initial ("day 0") and final ("day end") physical-chemical characteristics	3* + 3*
Enumeration of associated microflora or specific microflora	2 or 10 to 15

* one unit is enough if the FBO can demonstrate that products are homogeneous.

The whole experiment requires destructive sampling for microbiological procedures.

3.2.4.1 Preparation and inoculation of test units for determining μ_{\max}

Food

See "Food" in § 2.2.4.2

Inoculation procedure

For each curve, inoculate the test units with only one strain (not a mixture).

See "Inoculation procedure" in § 2.2.4.2

Contamination level

The contamination level should be targeted to be about 100 cfu/g.

3.2.4.2. Detection of *L. monocytogenes* in the foodstuff prior to the challenge test ("day 0") and enumeration at the end ("day end")

Prepare 6 test units to check for the absence of *L. monocytogenes* in the foodstuff, the so called "blank samples" are not inoculated.

If *L. monocytogenes* is detected in the "blank samples" at "day 0", the challenge test will be invalid, as exact starting numbers will not be known and a correct growth rate cannot be calculated. Enumeration results in the "blank samples" at "day end" have to be interpreted by the laboratory.

3.2.4.3. Determination of initial ("day 0") and final ("day end") physical-chemical characteristics

Prepare 6 test units to determine the physical-chemical characteristics: they are not inoculated with *L. monocytogenes*, but inject instead sterile physiological salt solution.

3.2.4.4. Enumeration of associated microflora

Enumerate the associated microflora in non-inoculated test units either at each sampling time or at “day 0” and “day end”.

3.2.5. Storage conditions for the inoculated foodstuff

Conduct the challenge test in this case at a fixed temperature, which is preferably close to the temperatures chosen for the prediction (see §2.2.5, and §3.2.10).

3.2.6. Measurement of physical-chemical characteristics

See § 2.2.6

3.2.7. Detection methods

See § 2.2.7

3.2.8. Enumeration methods

According to Annex I of Regulation No. 2073/2005, the reference enumeration method for *L. monocytogenes* is the standard method EN ISO 11290-2, amended. According to Article 5 of the same regulation, the use of alternative analytical methods is acceptable when the methods are validated against the reference method and if a proprietary method, certified by a third party in accordance with the protocol set out in EN/ISO standard 16140 or other internationally accepted similar protocols, is used. Other methods shall be validated according to internationally accepted protocols and their use authorised by the competent authority.

Associated microflora taken into account may be the mesophilic aerobic flora or a specific microflora of the food (e.g. lactic acid bacteria, *Pseudomonas*). To enumerate these flora, use methods in agreement with the Regulation (EC) No. 2073/2005. Methods used should follow relevant CEN, ISO or national standards for the organism and food type concerned.

3.2.9. Calculation of the maximum growth rate (μ_{\max})

Basic knowledge about predictive microbiology is necessary to interpret results, using the simplified approach suggested in the next two paragraphs.

Advanced knowledge about predictive microbiology is useful to use other models.

Calculate the results of the enumeration according to the Standard EN ISO 7218 and transform them in base-10 logarithm cfu/g (\log_{10} cfu/g).

The growth rate of each curve (i.e. all the experimental points from one batch) can be easily estimated by non-linear regression. Softwares as MicroFit (free of charge) can be used for that purpose. The MicroFit software (Figure 2) is based on the Baranyi model as primary model.

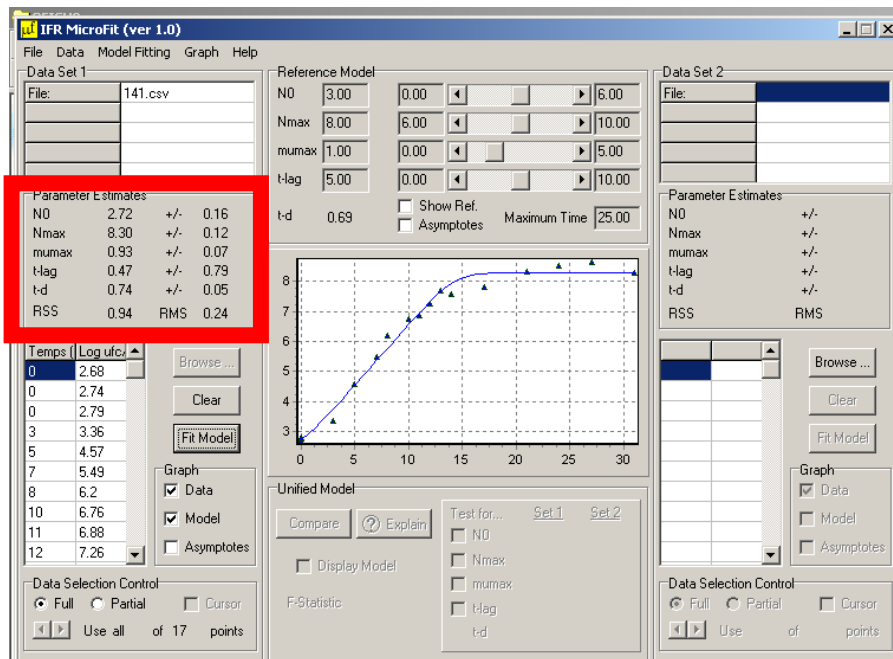


Figure 2. Use of Microfit software to fit a growth curve

The Microfit software provides a chart with the experimental points and the curve fitted by regression, using the Baranyi model. It also extracts the growth parameters of the curve: N_0 (logarithm of the initial bacterial concentration), N_{max} (logarithm of final bacterial concentration), μ_{max} (maximum growth rate), t_{lag} (lag time), t_d (generation time or doubling time). This generation time is related to the maximum growth rate by the relationship $t_d = \ln 2 / \mu_{max}$ (where "ln" stands for natural logarithm). The lag time and generation time are expressed in the time unit (e.g. days) and the maximum growth rate is then expressed in day^{-1} . Values of parameters are presented with their confidence interval. The software presents also the Residual Sum of Square (RSS) and the Root Mean Square (RMS). RSS and RMS give an indication of the quality of adjustment of the curve.

Choose the maximal value among the 6 μ_{max} obtained from each of the 6 growth curves for the further calculations.

3.2.10. Exploitation of the results

Knowing the value of μ_{max} at a temperature (T_{ref}), it is possible to calculate another μ_{max} at another temperature (T). Thus, from a growth curve at T_{ref} , the estimated μ_{max} using Microfit is denoted $\mu_{max_{ref}}$. Then, the calculation of μ_{max} in the same food (with the same physical-chemical characteristics) at another temperature T will be obtained using the square-root secondary model (Ratkowsky *et al.*, 1982; Zwietering *et al.*, 1996). If T and T_{ref} are both inferior to 25°C , the following simplified formula is suggested:

$$\mu_{max} = \mu_{max_{ref}} \cdot \frac{(T - T_{min})^2}{(T_{ref} - T_{min})^2}$$

(with T_{min} = minimal growth temperature for *L. monocytogenes* $\approx -2^\circ\text{C}$)

$\mu_{\max_{\text{ref}}}$ and μ_{\max} may be expressed in \log_{10} cfu/g per day by dividing their values by 2.3 [=ln(10)]

Other secondary models may be used.

Then, assuming a very simple primary model (without lag phase nor stationary phase, which may lead to fail-safe or even useless results):

Growth (in \log_{10}) obtained at T_{ref} during a time of storage d_1 (in days), is equal to $\mu_{\max_{\text{ref}}}$ (expressed in \log_{10} cfu/g per day) x d_1

Growth (in \log_{10}) obtained at a temperature T during a time of storage d_2 (in days), is equal to μ_{\max} (expressed in \log_{10} cfu/g per day) x d_2

Other primary models may be used.

The prediction can then be applied to any time-temperature profile, and in particular to the conditions at which the product is most likely to be subjected in normal use, until its final consumption (see § 2.2.5)

Example:

- **Data:**

- Shelf-life: 9 days,

- Storage conditions: 4°C for 3 days (d_1) and 8°C for 6 days (d_2)

The challenge test was performed at $T_{\text{ref}} = 8^\circ\text{C}$ and enabled to estimate $\mu_{\max_{\text{ref}}} = 0.78$ ln cfu/g per day, transformed into 0.34 \log_{10} cfu/g per day.

The secondary model enables to predict μ_{\max} at $T = 4^\circ\text{C}$, with its confidence interval

$$\mu_{\max} = \mu_{\max_{\text{ref}}} \cdot \frac{(T - T_{\min})^2}{(T_{\text{ref}} - T_{\min})^2}$$

The point-estimate is:

$$\mu_{\max} = \left[0.78 \frac{(4 - (-2))^2}{(8 - (-2))^2} \right] \text{ ln cfu/g per day} = 0.28 \text{ ln cfu/g per day transformed into } 0.12 \log_{10} \text{ cfu/g}$$

per day

Then, the growth rate predicted at 4°C is 0.12 \log_{10} cfu/g per day

- **Question 1:** what is the growth of *L. monocytogenes* predicted during the shelf-life?

Growth during the shelf-life =

$[(\mu_{\max_1} \text{ in log}_{10} \text{ cfu/g per day}) \times d_1] + [(\mu_{\max_2} \text{ in log}_{10} \text{ cfu/g per day}) \times d_2]$ where:

$$\text{Growth} = (3 \times 0.12) + (6 \times 0.34) = 2.40 \text{ log}_{10} \text{ cfu/g}$$

This calculation does not include the lag phase and the stationary phase (i.e. assumes the whole simulated behaviour is exponential growth), and consequently, the results may be (very) fail-safe or even useless.

- **Question 2:** what shall be the concentration at the beginning of the shelf-life in order to respect the limit of 100 cfu/g?

$$\diamond \text{ Initial concentration} = \text{Final concentration} - \text{growth during the shelf-life}$$

The final concentration is the limit of 100 cfu/g (2 log₁₀ cfu/g)

$$2 - 2.40 = -0.40 \text{ log}_{10} \text{ cfu/g} = 0.4 \text{ cfu/g}$$

- **Question 3:** what is the concentration of *L. monocytogenes* at the end of the shelf-life if the level for *L. monocytogenes* at day 7 is equal to 1.65 log₁₀ cfu/g?

The level of *L. monocytogenes* at "day end" will be $1.65 + 0.34 \times 2 = 3.33 \text{ log}_{10} \text{ cfu/g}$. The limit of 100 cfu/g is exceeded for this product.

3.2.11. Test report

Include in the test report at least the following information:

- ◇ Report number,
- ◇ Information concerning full identification of the foodstuff:
 - Identification of the batches tested and their manufacturing date,

- The characteristics of the foodstuff (pH, a_w , associated microflora, ...),
- ◇ Data relative to the strains under consideration:
 - Origin of the strains,
 - Inoculum preparation conditions,
 - Inoculum concentration,
- ◇ Data relative to the actual challenge test:
 - Batch numbers per product,
 - Number of test units,
 - Day of inoculation,
 - Mass or volume of the test units inoculated,
 - Inoculum volume and contamination method,
 - Storage conditions (time / temperature) of the test units,
 - Reference of the microbiological methods (enumeration and detection),
 - Threshold of the enumeration method,
 - Physical-chemical characteristics of the foodstuff at the beginning and the end of the test,
 - Level of associated microflora,
 - Raw data and calculations,
 - μ_{max} and interpretation.

4. Durability studies

To conduct durability studies the following points have to be considered: the sampling method, the storage conditions and the enumeration method for *L. monocytogenes*.

4.1. Food sampling

4.1.1. Introduction

The sampling procedure, under the responsibility of the FBO and with the collaboration of the laboratory who will conduct the durability study, must be representative of the population (to take into account the diversity of the population) and must have a good precision which depends on the size of the sampled sub-population.

Chapter 3, Clause 3.1 of Annex I to Regulation (EC) No. 2073/2005 refers to relevant ISO Standards and Codex Alimentarius Guidelines (CAC/GL 50-2004 "General guidelines on sampling") which shall be used as reference methods in the absence of more specific rules on sampling and preparation of tests samples.

When no information on the structure of the population is available, the most objective way to draw units is to give all the units of the population the same chance to be selected. The simple random sampling is recommended to estimate the proportion of units above the limit of 100 cfu/g.

4.1.2. The simple random sampling

This sampling method is based on the equiprobability principle. This principle guarantees each unit of the population to have an equal chance of being selected. To satisfy this principle, it is assumed that the size of the batch (N) must be large enough in comparison to the size (*n*) of the sampled sub-population: $n / N < 10\%$.

One way of achieving simple random sampling is to number the units or the production time and then to use random numbers to select the required sampled sub-population. For example, random numbers can be obtained from an Excel spreadsheet with the formula =RAND(), see Figure 3, or from random number tables.

Example of a method used to select units randomly

To draw randomly twenty units at the end the production line, the time is divided in periods of 5 minutes. An Excel spreadsheet is fulfilled with this sequence of 5 minutes and the random function selected gives to each sequence a random number. These random numbers are then sorted by increasing numbers and the first twenty ones are selected. Then, a person in charge of the sampling will draw at the end of the production line the twenty units at the selected times.

	A	B	C	D	E	F	G	H	I	J	K	L	M
1	Sequence (min)	Random number			Sequence (min)	Random number							
2	0	0.58848633			15	0.00930985		the first ten units will be pick out at					
3	5	0.02190713			120	0.01523129							
4	10	0.3612461			5	0.02190713		t= 5 min					
5	15	0.00930985			75	0.05645281		t=15 min					
6	20	0.6433244			70	0.06515071		t=25 min					
7	25	0.30029411			150	0.20429459		t=70 min					
8	30	0.39976866			145	0.21515049		t=75					
9	35	0.33929563			115	0.26105324		t=115					
10	40	0.43367777			25	0.30029411		t=120					
11	45	0.36069187			140	0.31117033	n=10	t=140					
12	50	0.56615491			130	0.33362289		t=145					
13	55	0.41870767			35	0.33929563		t=150					
14	60	0.33933409			60	0.33933409							
15	65	0.81140961			45	0.36069187							
16	70	0.06515071			10	0.3612461							
17	75	0.05645281			30	0.39976866							
18	80	0.63440726			55	0.41870767							
19	85	0.72503762			40	0.43367777							
20	90	0.80574511			95	0.43402673							
21	95	0.43402673			50	0.56615491	n=20						
22	100	0.78786226			0	0.58848633							
23	105	0.80661172			80	0.63440726							
24	110	0.94502085			20	0.6433244							
25	115	0.26105324			135	0.72172893							
26	120	0.01523129			85	0.72503762							
27	125	0.79779272			100	0.78786226							
28	130	0.33362289			125	0.79779272							
29	135	0.72172893			90	0.80574511							
30	140	0.31117033			105	0.80661172							
31	145	0.21515049			65	0.81140961							
32	150	0.20429459			110	0.94502085							

Figure 3. Example of a random sampling from an Excel spreadsheet

This sampling method should be repeated for different production days/batches (same product, produced under similar conditions) to obtain representative data.

4.2. Storage conditions

See § 2.2.5.

4.3. Microbiological analyses

At the end of the storage period, analyse all the units using the enumeration method in order to quantify if the level of 100 *Listeria monocytogenes* / g is exceeded or not.

According to Annex I of Regulation No. 2073/2005, the reference enumeration method for *L. monocytogenes* is the standard method EN ISO 11290-2, amended. According to Article 5 of the same regulation, the use of alternative analytical methods is acceptable when the methods are validated against the reference method and if a proprietary method, certified by a third party in accordance with the protocol set out in EN/ISO standard 16140 or other internationally accepted similar protocols, is used. Other methods shall be validated according to internationally accepted protocols and their use authorised by the competent authority.

The limit of enumeration should be 10 cfu/g, in order to be able to precisely quantify the contamination level of *L. monocytogenes* at the end of the storage period.

4.4. Calculation

In a routine practice of control (acceptability of a batch for example), the criterion defined by Regulation No. 2073/2005 is "n=5, c=0, m=M=100 cfu/g at the time of consumption". However, such controls are not in the scope of the present document.

The interpretation of durability studies, which consists in validating that the limit of 100 cfu/g is not exceeded at the time of consumption is a different case. As described below, it is suggested that is that this interpretation can be facilitated by the assessment of the proportion (with its associated confidence interval) of units exceeding 100 cfu/g at the end of the shelf-life, after a storage period reflecting the foreseeable conditions of distribution and storage.

From the sampled sub-population (of size n) taken randomly from a batch (of size N), deduce simply the estimated proportion of units exceeding 100 cfu/g at the end of the shelf-life as the observed proportion $p = r / n$ (where r is the number of units above 100 cfu/g, and n the size of the sampled sub-population).

To calculate the confidence interval associated to the estimated proportion, use a calculator. Numerous such calculators are freely available on the internet, for example: http://www.causascientia.org/math_stat/ProportionCI.html. This calculator proposed two methods of calculation, the central confidence interval or the shortest confidence interval. Confidence intervals given by each method may be slightly different but are in the same order of :magnitude.

On the sampled sub-population, after the storage period, the table below gives estimated proportions (p) with their confidence intervals for three r values (number of units > 100 *L. monocytogenes* / g).

Table 6 points out the real importance of drawing from the production line a sufficient number of units, and/or to gather results previously obtained, to estimate the proportion of units greater than 100 cfu/g with a reduced confidence interval.

Table 6. Example of estimated proportion of units > 100 *L. monocytogenes* / g after storage period

n number of analysed units	r number of units > 100 cfu/g	p estimated proportion	CI Confidence Interval at 95%
20	0	0%	[0% – 16%]
100		0%	[0% – 4%]
20	1	5%	[1% – 24%]
100		1%	[0.2% – 5%]
20	2	10%	[3% – 30%]
100		2%	[0.6% – 7%]

The more units that are analysed, the narrower the confidence interval; for example, it can be concluded from this table that the upper limit of the confidence interval for “2 units exceeding 100 cfu/g out of 100 units” is lower than that obtained for “0 units exceeding 100 cfu/g out of 20 units”.

To get a large number of analysed units, it is possible to gather results of repeated tests, performed on one RTE food obtained from the same process.

4.5 Test report

Include in the test report at least the following information:

- ◇ Report number,
- ◇ Information concerning full identification of the foodstuff:
 - Identification of the batches tested and their manufacturing date,
 - The characteristics of the foodstuff (pH, a_w , associated microflora, ...),
 - The intended shelf-life of the product,
- ◇ Justification of storage conditions (duration and temperature),
- ◇ Data relative to the durability studies conducted:
 - Batch numbers per product,
 - Number of test units,
 - Days of sampling,
 - Date of storage (beginning),
 - Storage conditions (time – temperature) of the test units,
 - Enumeration method – threshold of the method,
 - Estimated proportion of units above the limit of 100 cfu/g at the end of the durability study with its associated confidence interval.

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