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EURL *Lm*
European Union Reference Laboratory for *Listeria monocytogenes*

EURL *Lm* Guidance Document to evaluate the competence of laboratories implementing challenge tests and durability studies related to *Listeria monocytogenes* in ready-to-eat foods

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Foreword

This guidance document has been prepared by the European Union Reference Laboratory for *Listeria monocytogenes* (EURL *Lm*), in collaboration with representatives of 7 National Reference Laboratories for *Listeria monocytogenes* (NRLs *Lm*).

This is the second version of this guidance document, replacing the first version of February 2012. This 2nd version takes into account the 2014 version of the EURL *Lm* Technical guidance document for conducting shelf-life studies on *Listeria monocytogenes* in ready-to-eat foods, as well as experiences gained from the evaluation of shelf-life studies.

This document has been approved by the EC Standing Committee on Plants, Animals, Food and Feed (PAFF Committee) at its meeting of 3 May 2018.

1 INTRODUCTION

1.1 Legislative background

Regulation (EC) No 178/2002 of 28 January 2002[1] lays down the general principles governing food in general, and food safety in particular, at Community and national level. This regulation also lays down procedures for matters with a direct or indirect impact on food safety. Article 14 that sets out the food safety requirements: "food must not be placed on the market if it is unsafe", meaning "injurious to health" or "unfit for human consumption". This regulation also sets out responsibilities of food business operators (FBOs) and establishes the principle that the primary responsibility for ensuring compliance with food law lies on FBOs.

Regulation (EC) No 2073/2005[2] of 15 November 2005 on microbiological criteria for foodstuffs sets out specific food safety criteria for *L. monocytogenes* in ready-to-eat (RTE) foods (category 1.1 to 1.3 of Annex I of this Regulation). For RTE foods, other than those intended for infants and for medical purposes, which are able to support the growth of *Listeria monocytogenes* (category 1.2), two microbiological criteria are laid down: either a qualitative criterion (absence in 25g before the food has left the immediate control of the FBO who has produced it) or a quantitative criterion (limit of 100 cfu/g for products placed on the market during their shelf-life). This quantitative criterion applies if the FBO is able to demonstrate, to the satisfaction of the competent authority, that its product will not exceed the limit of 100 cfu/g throughout the shelf-life. To do so and according to Article 3.2, the FBO shall conduct studies referred in Annex II of this Regulation.

[1] OJ L 31, 01.02.2002, p.1
[2] OJ L 338, 22.12.2005, p.1.

1.2 Scope

Two European guidance documents for the implementation of Regulation (EC) 2073/2005 have been published. One is mainly directed at FBOs, in order to guide them in identifying the *L. monocytogenes* risk in their RTE foods, while the other one is dedicated to laboratories in order to help them in implementing shelf-life studies:

- "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", DG-SANCO 1628/2008;
- "EURL *Lm* Technical guidance document for conducting shelf-life studies on *Listeria monocytogenes* in ready to eat foods", version 3 – 6 June 2014**.**

The aim of the present guidance document is to set up a harmonized approach to evaluate the competence of laboratories conducting shelf-life studies (challenge tests and durability studies), in order to comply with the food safety criteria defined in (EC) Regulation No 2073/2005 modified.

This document is intended for use by national Competent Authorities (CAs), NRLs and other organizations that are involved in assessing whether laboratories are competent to conduct shelf-life studies related to *Listeria monocytogenes*. This assessment may be undertaken through an audit, or based on a shelf-life study report.

Regarding more precisely the use of this document by CAs, it can serve as a tool for CAs to evaluate the implementation of foot-note 5 to *Lm* criterion 1.2 of Regulation (EC) 2073/2005 modified, which specifies that manufacturer shall be able to demonstrate, **to the satisfaction of the competent authority**, that the product will not exceed the limit 100 cfu/g throughout the shelf-life.

The first part (Chapter 2) of this document focuses on the expertise needed to design, conduct and interpret a study. Its second part (Chapter 3) deals with the technical competence of the laboratory.

1.3 References

The following referenced documents are indispensable for the application of this document. The most recent edition of the standard shall be used.

- EN ISO 6887 -1, Microbiology of the food chain -- Preparation of test samples, initial suspension and decimal dilutions for microbiological examination -- Part 1: General rules for the preparation of the initial suspension and decimal dilutions.
- EN ISO 6887 -2, Microbiology of the food chain -- Preparation of test samples, initial suspension and decimal dilutions for microbiological examination -- Part 2: Specific rules for the preparation of meat and meat products.
- EN ISO 6887 -3, Microbiology of the food chain -- Preparation of test samples, initial suspension and decimal dilutions for microbiological examination -- Part 3: Specific rules for the preparation of fish and fishery products.
- EN ISO 6887 -4, Microbiology of the food chain -- Preparation of test samples, initial suspension and decimal dilutions for microbiological examination -- Part 4: Specific rules for the preparation of products other than milk and milk products, meat and meat products, and fish and fishery products.
- EN ISO 6887 -5, Microbiology of the food chain -- Preparation of test samples, initial suspension and decimal dilutions for microbiological examination -- Part 5: Specific rules for the preparation of milk and milk products.
- EN ISO 7218, Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations.
- EN ISO 11290-1, Microbiology of the food chain – Horizontal method for the detection and enumeration of *Listeria monocytogenes* and of *Listeria* spp.– Part 1: Detection method.
- EN ISO 11290-2, Microbiology of food chain – Horizontal method for the detection and enumeration of *Listeria monocytogenes* and of *Listeria* spp.– Part 2: Enumeration method.
- EN ISO 16140-2, Microbiology of the food chain — Method validation — Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method.
- ISO 17025, General requirements for the competence of testing and calibration of laboratories.
- ISO 18787, Foodstuffs – Determination of water activity
- ISO 21807, Microbiology of food and animal feeding stuffs - Determination of water activity.
- EURL *Lm* Technical guidance document for conducting shelf-life studies on *Listeria monocytogenes* in ready-to-eat-foods.

  https://ec.europa.eu/food/sites/food/files/safety/docs/biosafety_fh_mc_technical_guidance_document_listeria_in_rte_foods.pdf
- 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, Commission of the European Communities.

  https://ec.europa.eu/food/sites/food/files/safety/docs/biosafety_fh_mc_guidance_document_lysteria.pdf
- Regulation (EC) No. 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs, amended.

1.4 Challenge test assessing the growth potential of *L. monocytogenes*

A challenge test assessing the growth potential is a microbiological laboratory-based study that measures the growth of *L. monocytogenes* in artificially contaminated food stored under reasonably foreseeable conditions from production to consumption (storage at producer, distribution, storage at retail and at consumer level). This growth potential (δ) is defined as the difference between the $\log_{10}$ cfu/g at the end of the challenge test and the $\log_{10}$ cfu/g at the beginning of the test. For further details, refer to the guidance documents related to shelf-life studies of ready-to-eat foods.

To comply with the microbiological criteria for *Listeria monocytogenes* set out in Annex I (food categories 1.2 and 1.3) of Regulation (EC) No. 2073/2005, the growth potential (δ) at a given time-temperature scenario can be used:

- To classify a food:
  - in category 1.2 "Ready-to-eat foods able to support the growth of *L. monocytogenes*" when $\delta > 0.5$ $\log_{10}$ cfu/g.
  - in category 1.3 "Ready-to-eat foods unable to support the growth of *L. monocytogenes"* when $\delta \leq 0.5$ $\log_{10}$ cfu/g,
- To assess the growth of *L. monocytogenes* in a RTE food classified in category 1.2, according to defined reasonably foreseeable conditions of storage between production and consumption.

The growth potential (δ) depends on many factors, the most important being:

- intrinsic properties of the food (e.g. pH, NaCl content, water activity (a_w), intrinsic microflora, preservatives),
- extrinsic properties (e.g. time-temperature profile, packaging conditions),
- physiological state of the inoculated strain(s), and level of contamination.

1.5 Challenge test assessing maximum growth rate of *L. monocytogenes*

A challenge test assessing the maximum growth rate is a microbiological laboratory-based study that measures the growth rate of *L. monocytogenes* in artificially contaminated food stored at a defined temperature. For further details, refer to the guidance documents related to shelf-life studies of ready-to-eat foods.

The maximum growth rate (μ_{max} in natural logarithm) is calculated from the exponential phase of a growth curve of *L. monocytogenes* obtained at a defined temperature by plotting the natural logarithm of the bacterial population versus time. The slope of the line in this phase is the μ_{max}.

The maximum growth rate is an important parameter of the bacterial growth kinetic which depends on:

- the inoculated strain,
- intrinsic properties of the food (e.g. pH, $a_{w,}$ water content, NaCl content, intrinsic microflora, antimicrobial constituents),
- extrinsic properties (e.g. temperature, gas atmosphere).

Maximum growth rate can be estimated by linear regression or non-linear regression, and be used to directly calculate an increase in bacterial counts and/or used in predictive microbiological software.

1.6 Durability study

A durability study is a microbiological study used to determine the evolution of bacterial populations naturally present in a food stored under reasonably foreseeable conditions from production to consumption (storage at producer, distribution, storage at retail and at consumer level).

2 ASSESSMENT OF THE LABORATORY EXPERTISE

2.1 Requirements related to the laboratory

The laboratory performing shelf-life studies shall have, or else have access to relevant knowledge in food microbiology, food sciences and technology, predictive models in microbiology and statistics necessary to design and conduct the studies, to interpret the results and draw conclusions..

Knowledge from the FBO on their products shall be combined with the knowledge of the laboratory outlined in the previous paragraph to ensure the robustness of the study.

The responsibility of the laboratory is to design shelf-life studies based on the information from the FBO. The FBO is responsible for providing data about the products and the storage conditions (time-temperature profile) relevant where the product is sold, while the laboratory needs to have sufficient competence to give guidance based on information received from the FBO. The laboratory should have knowledge of Commission Regulation (EC) No 2073 / 2005 of 15 November 2005 on microbiological criteria for foodstuffs amended (Regulation (EC) No 2073/2005), 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 (DG – SANCO Guidance document) and EURL *Lm* Technical guidance document for conducting shelf-life studies on Listeria monocytogenes (EURL *Lm* Technical guidance document), or of the standards on challenge tests or durability studies applied at national level, if existing.

The laboratory and the person responsible for the study should be identified. The staff involved in the studies should be able to provide evidence of competence, for instance be trained in implementing or have documented experience in challenge tests.

All the analytical methods (microbiological) used to perform challenge test and durability study should be specified and according to Annex I of Regulation (EC) No 2073/2005, be performed using standardized reference methods or accepted alternative methods according to Article 5 of this regulation.

It is **recommended** that the laboratory is accredited for:

- detection and enumeration of *L. monocytogenes* in food. The methods used shall fulfill the requirements specified in Article 5 of Regulation (EC) No 2073/2005.

- measurement of physico-chemical parameters (i.e. water activity and pH) and microbiological analyses, such as enumeration of bacteria useful for interpretation of the results of the challenge test.

If the laboratory is not accredited for these methods, the minimum quality assurance level expected is to have documented good laboratory practices, perform own metrological quality control tests and to have successfully participated in proficiency tests.

To apply predictive microbiological models, the laboratory should have knowledge on the available software packages and have the ability to use the relevant software for the products tested.

2.2 Review of information provided by the FBO to the laboratory

2.2.1 General

Product information for conducting shelf-life studies is stated in the DG-SANCO Guidance document. The FBO should provide relevant product information (e.g. shelf-life, range of pH, range of a_w, storage temperature, historical data for the growth of *L. monocytogenes* in the product and/or from previous shelf-life studies). The laboratory can also provide product information (in particular pH and a_w) based on analyses performed on the product before starting a challenge test. The laboratory shall critically evaluate the obtained information (at least the ones listed below). The laboratory shall advise the FBO of the relevance of implementing or not a durability study or a challenge test for the specific RTE food and shall, based on the information obtained, design the experimental protocol of the challenge test.

2.2.2 Information required for designing a challenge test

- **Selection of the batches and of the product**

The batches should be representative of the variability of the manufacturing conditions. If the challenge test is related to a range of products, only the product which is expected to give the worst case scenario for *L. monocytogenes* growth needs to be tested. The selection of worst case product should be justified in the test report.

These following items should be considered.

- **The study is relevant for:**
  - a single product;
  - a product representing a range of products.
- **Information on the product label**
  - Name of the product or identifiable code for new product development;
  - Storage temperature;
  - Weight of the product;
  - List of ingredients;
  - 'Use by' date or assigned date for new product;
  - Photograph of the product and label.
- **Shelf-life of the product**
  - Production date;
  - Shelf-life (used by date or assigned date for new product).
- **History of the product**
  - New product, new formulation;
  - Product commercialized
- **Production process**
  - Main steps (linked to the inactivation of microorganisms or to possible recontamination).
- **Packaging of the product**
  - Under air;
  - Under vacuum;
  - Under modified atmosphere (gas composition);
  - Properties of packaging material (e.g. permeability, …).
- **Physico-chemical characteristics of the product**
  - Data sets (number of values, period covered) for parameters of concern;
  - Parameters of concern (including the mean, standard deviation and the range):
    - pH of the product;
    - aw of the product or water phase salt (WPS);
    - optional: salt, fat, sugar content, concentration of preservatives.
- **Microbiological characteristics of the product:**
  - Data sets for L. monocytogenes (number of values, period covered, prevalence, level of contamination, data exceeding the limit of 100 cfu/g);
  - Data sets for microorganisms (other than *L. monocytogenes*) of significance (number of values, period covered, level of contamination):
    - Total microflora, Lactic bacteria, … ;
    - Technological microflora (addition of probiotics, starter cultures, ...).

In case of new products, the FBO and the laboratory should, as a minimum, make an estimate of all above mentioned items, based on the ingredients, process conditions and similar products.

- **Characterisation of the cold chain:**
  - Storage temperature and duration : at the production, from production to retail, at retail, at consumer level;
  - Data sets (origin and number of values, period covered, 75th percentile);
  - Destination of the product (national marketplace and/or other EU member states).

When data are not available on storage temperature and duration, the laboratory must use default values specified in the EURL *Lm* Technical guidance document.

2.3 Report of the shelf-life studies

At the end of the study, the laboratory shall provide a report that sets out the conditions under which the study has been carried out. For challenge tests, it shall include at least the information listed in the chapter 3.2.1.2 section k or chapter 3.2.2.2 section g of the EURL *Lm* Technical guidance document.

In addition, the report shall have a section dedicated to the results and a section for the conclusion including the applicability and the limitation of the study.

3 ASSESSMENT OF THE TECHNICAL COMPETENCE OF THE LABORATORY

3.1 Challenge tests

The following items, identified in the EURL *Lm* Technical guidance document for conducting shelf-life studies on *L. monocytogenes* in ready to eat foods should be specified in the challenge testing experimental design.

3.1.1 Number of batches

Three batches will be tested in general. The laboratory must analyse batches selected at different times to take into account the between-batch variability. The three batches should represent the variation in the production process and ingredients. When less than three batches are tested, the reason should be justified in the report.

To determine the number of batches to be tested, the laboratory shall use:

- for growth potential, a recognized and commonly accepted "growth /no growth boundary" model (e.g EURL *Lm* Technical guidance document section 3.2.1.2);
- for growth rate, the inter-batch variability calculator (https://eurl-listeria.anses.fr) or a recognized and commonly accepted "growth /no growth boundary" model (e.g EURL Lm Technical guidance document section 3.2.1.2).

3.1.2 Strains

To consider the variability among strains, it is recommended that the laboratory conduct the challenge test with several strains.

- Number of strains:
  - At least 2 strains must be used;
  - The origin of the strains shall be given (including the product from which the strain was isolated if known);
  - The growth characteristic of one of these strains must be documented;
  - Depending on the challenge test performed, these strains must be used in a mixture for the growth potential or individually for the maximum growth rate.

3.1.3 Preparation of the inoculum

The laboratory shall perform the preparation of the inoculum to avoid introducing as much as possible a bias when artificially inoculating *L. monocytogenes* in the product.

- Number of subcultures:
    - Two successive subcultures must be performed in an appropriate medium, until reaching the early stationary phase. Incubate at the optimal growth temperature (first subculture) and at or close to a temperature for the storage temperature of the product in the first step in the cold chain of the product (second subculture);
    - For mixed cultures (growth potential), equal quantities of each second subculture shall be mixed.
- Inoculum:
    - The target inoculum concentration shall be obtained by diluting mixed culture (growth potential) or second subculture (maximum growth rate) in physiological water;
    - The inoculum should be used immediately and its concentration checked on the selective agar used for the test.

3.1.4 Inoculation of test units

Based on the collected information provided by the FBO, the laboratory shall choose between the available methods listed in the EURL *Lm* Technical guidance document:

- Surface or in-depth inoculation;
- With or without de-packaging of the product.

The laboratory shall justify the relevance of the inoculation method for the product studied.

The laboratory shall use suitable equipment (*e.g.* septum and syringe) to inoculate the products.

The targeted level of contamination (around 100 cfu/g) shall be respected as well as the volume of inoculation (≤ 1% of the mass of the test unit inoculated).

3.1.5 Storage of test units

This step is of major importance, especially in challenge testing assessing the growth potential. The combinations of temperature/duration for each step of the cold chain shall be justified according to table 3 of section 3.2.1.2. of the EURL *Lm* Technical guidance document:

- Time/temperature profile supported by information provided by the FBO (75th percentile of own FBO's data observation);
- Time/temperature profile based on national data (75th percentile, where the cold chain occurs);
- Time/temperature profile defined as default values (8°C, 12°C, 12°C).

The laboratory shall give evidence that test units are stored under the time /temperature profile defined in the protocol.

3.1.6 Physico-chemical measurements of un-inoculated test units

To characterize the product on which the challenge test is performed, the laboratory shall measure, on un-inoculated test units, physico-chemical parameters, such as:

- pH, a_w, or NaCl and moisture content;
- Gas composition;
- Other parameters.

The laboratory shall specify when these analyses are performed and on how many un-inoculated test units (coming from the same batches as the inoculated products) these measurements are carried-out. At least one sample shall be used at the beginning and one sample at the end of the study per batch.

3.1.7 Microbiological analyses

The methods used shall fulfill the requirements specified in Article 5 of Regulation (EC) No 2073/2005.

To assess the behavior of *L. monocytogenes* artificially introduced in the product, the laboratory shall enumerate the concentration of *L. monocytogenes* using the reference method EN ISO 11290-2 or an alternative method validated according to EN ISO 16140-2.

The laboratory shall ensure that the lower limit of enumeration is 10 cfu/g.

To be sure that the challenge test is performed on products that are free of *L. monocytogenes*, the laboratory shall carry out the detection of *L. monocytogenes* in un-inoculated test units using the reference method EN ISO 11290-1 or an alternative method validated according to ISO 16140-2.

Per batch tested, at least one sample shall be used at the beginning and one sample at the end of the study. If *L. monocytogenes* is detected in the samples, the laboratory must inform the FBO immediately,

and if the challenge test has been completed (detection of *L. monocytogenes* at the end of the study), where the data are used for evaluation of the growth potential, a justification shall be provided in the report.

To characterize the product of concern, the laboratory should enumerate, using un-inoculated test units, the natural microflora relevant for the product: e.g total microflora, lactic bacteria or yeasts, but at least total microflora.

The laboratory shall document when these analyses are conducted and on how many un-inoculated test units these analyses are performed. Per batch tested, at least one sample shall be used at the beginning and one sample at the end of the study.

3.1.8 Determination of the growth potential and exploitation of the results

To calculate the growth potential of *L. monocytogenes* of the studied product, the laboratory shall:

- Determine the concentration of *L. monocytogenes* (in $\log_{10}$ cfu/g) at the beginning and at the end of the challenge test using three samples per batch, as described in the EURL *Lm* Technical guidance document;
- Check for each batch that at day 0, the standard deviation of *L. monocytogenes* enumerations is ≤ 0.5 $\log_{10}$ cfu/g. If not the case, the challenge test is inconclusive;
- Use the calculation of the growth potential provided in the EURL *Lm* Technical guidance document (section 3.2.1.2.);
- Select, among the growth potential obtained for each batch, the highest value as the final outcome of the study.

Based on the obtained results, the laboratory shall be able to conclude to a significant or a non-significant increase of *L. monocytogenes* in the studied product. When the difference between the maximum and minimum of the 3 values of one batch is high, the laboratory shall notify this information in the test report along with the expert recommendations from the study.

In addition, any observation that could influence the validity of the data or conclusion must be reported.

3.1.9 Determination of the maximum growth rate and exploitation of the results

To calculate the maximum growth rate of *L. monocytogenes* in the studied product, the laboratory shall:

- Build the growth curves of *L. monocytogenes* (concentration of *L. monocytogenes* in $\log_{10}$ cfu/g *versus* time) at one defined temperature for two strains, individually tested;
- Apply a linear regression on experimental data points in the exponential phase, or fit a non-linear regression to all the experimental data points using a microbiological software;
- The laboratory shall be able to give a confidence interval for the growth rate according to standard error given by the software, and select among the maximum growth rate obtained for each batch, the highest value as the final outcome of the study.
- The laboratory shall be able to extrapolate the μ_{max} obtained in the study to another temperature using the formula for secondary model given in the EURL *Lm* Technical guidance document.

3.2 Durability studies

Durability studies are carried out on batches that are likely to be naturally contaminated by *L. monocytogenes.* These studies differ from challenge tests as samples are not artificially contaminated. Due to high heterogeneity of *L. monocytogenes* contamination in batches, the random selection of samples is essential as not all samples may be contaminated. Assessment of product characteristics, shelf-life and cold storage conditions for durability studies are important to consider.
In durability studies, the number of samples above 100 cfu/g can be assessed in terms of frequency and trends.

3.2.1 Food sampling procedure

The laboratory should request from the FBO historical data (prevalence of *L. monocytogenes*) to be able to give advice on the value of performing or not a durability study. The laboratory should be able to give guidance to the FBO about sampling procedures for random and targeted sampling, and take the sampling procedure into account in interpretation of the results. For analyses of more batches, the distribution in time between batches should be given.

3.2.2 Storage conditions and analyses of parameters

See experimental challenge testing procedures (§ 3.1.5 to § 3.1.7).

3.2.3 Calculation and exploitation of the results

The percentage of samples above 100 cfu/g should be calculated and expressed with a confidence interval. This confidence interval can easily be obtained using a software, *e.g.* http://www.causascientia.org/math_stat/ProportionCI.html .

All measured data for *L. monocytogenes* should be given in the report to allow further calculations of the data.

In case of samples above 100 cfu/g by the end of shelf-life, the laboratory should inform the FBO and the date for such information should be included in the test report.

ANNEX 1 - Definitions

Batch
A 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.

Challenge test
Study of the evolution of a bacterial population artificially inoculated in a food

Cold chain
The continuous system that provides chilled storage of perishable foods, from production to consumption

Durability study
Study of the evolution of a bacterial population naturally present in a food

Growth potential
Difference between the log10 of the concentration of the artificially inoculated bacterial population at the defined end of a challenge test and the log10 of its initial concentration

Maximum growth rate
Slope of the curve showing the evolution of the natural logarithm of the population according to the time during the exponential phase

pH
A measure of the acidity or alkalinity of a food. The pH 7 is defined as neutral. Values of a pH less than seven are considered acidic and those with greater than seven are considered basic (alkaline).

Ready-to-eat (RTE) food
Food intended by the producer or the manufacturer for direct human consumption without the need for cooking or other processing effective to eliminate or reduce to an acceptable level, microorganisms of concern.

Shelf-life
Either the period corresponding to the period preceding the 'use by' or the minimum durability date, as defined respectively in Articles 9 and 10 of Directive 2000/13/EC concerning, among others, the labelling of foodstuffs.

Water activity (a_w)
The term refers to the unbound and available water in a food and is not the same as the water content of the food. Water in food which is not bound to other molecules can support the growth of microbes. The water activity scale extends from 0 to 1.0 (pure water) but most foods have a water activity level in the range of 0.2 for very dry foods to 0.99 for moist fresh foods.

ANNEX 2 - Example of a check list to assess the technical competence of the laboratory performing a challenge test

The following items, identified in the "EURL *Lm* Technical guidance document for conducting shelf-life studies on *Listeria monocytogenes* in ready to eat foods", version 3 – 6 June 2014, should be specified in experimental design for challenge test.

	Specifications of EURL *Lm* Technical Guidance Document	Information provided Yes	No	Comment
Number and choice of the batches				
Determining the number of batches to be tested	Use of a predictive microbiological software. Growth/No growth boundary.	☐	☐	
	Use of the inter-batch variability calculator.	☐	☐	
Number of batches to be tested	• At least 3 batches.	☐	☐	
	• 1 batch, if growth probability ≤10%.	☐	☐	
	• 1 batch, if inter-batch variability insignificant for Lm.	☐	☐	
Selection of the batches (if at least 3 batches tested).	Batches with the physico-chemical characteristics the most favorable to growth.	☐	☐	
	Batches spread over time	☐	☐	

	Specifications of EURL *Lm* Technical Guidance Document	Information provided Yes	No	Comment
Strains				
Number of strains	At least 2 strains.	☐	☐	
Selection of the strains	1 strain with known growth characteristics and	☐	☐	
	other (s) strain(s) freely chosen (for example : environment, outbreak ...)	☐	☐	
Use	In mixture	☐	☐	
	Individually	☐	☐	
Control of the strains (biochemical, genoserotype, growth)	Control procedure	☐	☐	

	Specifications of EURL *Lm* Technical Guidance Document	Information provided Yes	No	Comment
Preparation inoculum				
Preparation of the subcultures	1st subculture in broth (TSB or BHI) at 30°C or 37°C until to reach the stationary phase.	☐	☐	
	2nd subculture at a T° near the storage T° of the product, until to reach the early stationary phase.	☐	☐	
Mixture of the subcultures	In equal quantities.	☐	☐	
Dilution of the mixture or 2nd subculture	In physiological water	☐	☐	
Use of the inoculum	Immediately	☐	☐	
Enumeration of the inoculum	On selective agar	☐	☐	

	Specifications of EURL *Lm* Technical Guidance Document	Information provided Yes	No	Comment
Inoculation of the samples				
Initial level of contamination	Targeted level around 100 cfu/g.	☐	☐	
Inoculum volume	Volume of the inoculum ≤ 1% of the mass of the sample inoculated.	☐	☐	
Methods of contamination	• Depackaged products: - in depth (for homogeneous foods or homogenized after grinding) or	☐	☐	
	- on the surface (to simulate contamination during the process).	☐	☐	
	For foods with multicomponents : - contamination of the part likely contaminated with Lm.	☐	☐	
	- contamination at the interface of ingredients.	☐	☐	
	Repackaging in the initial condition: gaseous composition, gas volume	☐	☐	
	• Packaged products: - on the surface through a septum	☐	☐	
	- double septum.	☐	☐	

	Specifications of EURL *Lm* Technical Guidance Document	Information provided Yes	No	Comment
Storage of the samples				
Time /storage temperatures (growth potential determination)	• Justified from detailed information given by the FBO	☐	☐	
	• Justified from data available at national level	☐	☐	
	• Use of the values given in table 3 of the EURL Lm Technical Guidance	☐	☐	
Storage temperature(growth rate determination)	At a fix temperature	☐	☐	

	Specifications of EURL *Lm* Technical Guidance Document	Information provided Yes	No	Comment
Physico-chemical measurements				
Parameters measured and number of samples analysed.	*pH measurement on at least 1 sample at Day_0 and at Day_{end}.	☐	☐	
	According to a national or international standard method	☐	☐	
	* a_w measurement on at least 1 sample at Day_0 and t Day_{end}.	☐	☐	
	According to a national or international standard method	☐	☐	
	* Measurement [NaCl] and water content on at least 1 sample (at Day_0 and at Day_{end})	☐	☐	
	* Measurement of the gaseous atmosphere at Day_0 and at Day_{iend}	☐	☐	
	* Other measured parameters	☐	☐	
	Addition of a volume of physiological water equivalent to the inoculum volume	☐	☐	

	Specifications of EURL *Lm* Technical Guidance Document	Information provided Yes	No	Comment
Microbiological Analyses				
Detection Lm	On at least 3 uninoculated samples at Day_0 and Day_{end}	☐	☐	
	according to :			
	- reference method EN ISO 11290-1	☐	☐	
	- alternative method validated according to ISO 16140-2	☐	☐	
Enumeration Lm	On at least 3 inoculated samples at Day_0 and Day_{end}	☐	☐	
	according to :			
	- reference method EN ISO 11290-2	☐	☐	
	- alternative method validated according to ISO 16140-2	☐	☐	
	Lower enumeration limit at 10 cfu/g.	☐	☐	
	Verification of the homogeneity of the contamination in *Lm* at Day0 ($\sigma \leq 0.5$).	☐	☐	
Enumeration of the total microflora	On at least 1 uninoculated sample, according to a national or international standard method	☐	☐	
Enumeration of a specific microflora (recommended)	On at least 1 uninoculated sample, according to a national or international standard method	☐	☐	

	Specifications of EURL *Lm* Technical Guidance Document	Information provided Yes	No	Comment
Determination of the growth potential				
Calculation method	Calculation of the Lm concentration in log_{10} at Day_0 and at Day_{end}	☐	☐	
	Calculation formula used for δ : Median of the results at Day_{end} – Median of the results at Day_0	☐	☐	
	Determination of δ for each batch	☐	☐	
	The highest δ value is retained	☐	☐	
	Significant increase of Lm (δ > 0.5 Log cfu/g).	☐	☐	
Determination of the growth rate				
Calculation method	Determination of the growth rate for each batch.	☐	☐	
	Lm concentrations expressed in Log_{10} cfu/g and building of the growth curves of Lm	☐	☐	
	Calculation of the growth rate : By linear regression on at least 5 dates and with 3 enumerations by date of analysis.	☐	☐	
	By non-linear regression using a primary model of a predictive microbiological software	☐	☐	
	Calculation of the confidence interval	☐	☐	
	The growth rate retained is the upper value of the confidence	☐	☐	
Extrapolation of the results	Extrapolation of the growth rate obtained at the studied T°, to growth rate at other T°	☐	☐	
	Determination of the growth of Lm at any realistic time-temperature profile, until the end of the shelf-life of the product.	☐	☐	

	Specifications of EURL *Lm* Technical Guidance Document	Information provided Yes	No	Comment
Test report				
Purpose of the study		☐	☐	
Type of challenge-test		☐	☐	
Identification of the food	Name of the product or identifiable code for new product development (NPD)	☐	☐	
	Shelf-life or assigned date for NPD	☐	☐	
	Characteristics of the product (physico – chemical and microbiological)	☐	☐	
	Identification of the batches	☐	☐	
Data relating to the challenge test	Number of batches tested	☐	☐	
	Number of tested units par batch	☐	☐	
	Mass of volume of the test units	☐	☐	
	Strains used	☐	☐	
	Preparation of the inoculum	☐	☐	
	Inoculum concentration	☐	☐	
	Volume of inoculum introduced per test unit	☐	☐	
	Contamination method	☐	☐	
	Date(s) of inoculation	☐	☐	
	Duration of the test and sampling interval	☐	☐	
	Storage temperature/duration and justification	☐	☐	
	Enumeration and detection method used	☐	☐	
	Limit of enumeration	☐	☐	
	Physico-chemical values at Day_0 and at Day_{end}	☐	☐	
	Gas atmosphere	☐	☐	
	Concentration of the associated microflora at Day_0 and Day_{end}	☐	☐	
	Concentration of the *L. monocytogenes* at Day_0 and Day_{end}	☐	☐	
	Growth potential per batch	☐	☐	
	Growth rate per batch	☐	☐	
	Conclusion	☐	☐	