EURL *Lm*

European Union Reference Laboratory for *Listeria monocytogenes*

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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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CONTENT

FOREWORD

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

This is the third version of the 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. It replaces version 2 of May 2018.

The purpose of this revision is to ensure consistency with version 4 of the EURL *Lm* Technical guidance document on challenge tests and durability studies for assessing shelf-life studies of ready-to-eat foods related to *Listeria monocytogenes* (*Lm*), as well as experiences gained from the evaluation of shelf-life studies.
This document was endorsed by the section Biological Safety of the Food Chain of the EC Standing Committee on Plants, Animals, Food and Feed (PAFF Committee) at its meeting of 10 February 2023.

1. INTRODUCTION

1.1. Legislative background

Regulation (EC) No 178/2002 lays down the general principles governing food in general, and food safety in particular, at Community and national level. 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 on microbiological criteria for foodstuffs sets out specific food safety criteria for *L. monocytogenes (Lm)* 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 *Lm* (category 1.2), two microbiological criteria are laid down: either a qualitative criterion (not detected in 25g before the food has left the immediate control of the FBO who has produced it) or a quantitative criterion (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 - paragraph 2, the FBO shall conduct, as necessary, studies referred in Annex II of this Regulation to evaluate the growth of *Lm* that may be present in the products during their shelf-life under reasonably foreseeable storage conditions.

1.2. EU Guidance documents

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

(i) "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", EC/DG-SANCO document;

(ii) "EURL *Lm* Technical guidance document on challenge tests and durability studies for assessing shelf-life of ready to eat foods related to *Listeria monocytogenes*."

2. SCOPE

This guidance document provides guidelines on how to evaluate the competence of laboratories conducting shelf-life studies: challenge tests and durability studies, in order to have a harmonised evaluation approach in the EU.

The evaluation is based on the expertise of the laboratory (e.g. knowledge, personnel) and on the technical competence of the laboratory (e.g. equipment, analytical methods). It is intended to be used by national Competent Authorities (CAs), accreditation bodies, NRLs, other organisations, if mandated by their CAs, involved in assessing competency to conduct shelf-life studies related to *Lm*. This assessment may be undertaken through an audit, or based on shelf-life study reports.

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 amended, which specifies that manufacturer shall be able to demonstrate, **to the satisfaction of the competent authority**, that the product will not exceed the limit of 100 cfu/g throughout the shelf-life.

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.
- EN ISO 20976-1, Requirements and guidelines for conducting challenge tests of food and feed products - Part 1: challenge tests to study the growth potential, lag time and maximum growth rate.
- 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 on challenge tests and durability studies for assessing shelf-life of ready to eat foods related to *Listeria monocytogenes*.
  https://ec.europa.eu/food/system/files/2021-07/biosafety_fh_mc_tech-guide-doc_listeria-in-rte-foods_en_0.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, EC/ DG SANCO.
  https://ec.europa.eu/food/system/files/2016-10/biosafety_fh_mc_guidance_document_lysteria.pdf
- Regulation (EC) No. 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs, amended.

4. SHELF-LIFE STUDIES

4.1. Challenge test assessing the growth potential of *Lm*

A challenge test assessing the growth potential is a microbiological laboratory-based study that measures the growth of *Lm* 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 highest $\log_{10}$ cfu/g during the challenge test and the initial *Lm* concentration in $\log_{10}$ cfu/g at the beginning of the test. For further details, refer to the standard EN ISO 20976-1 on "Requirements and guidelines for conducting challenge tests of food and feed products - part 1 : Challenge tests to study growth potential, lag time and maximum growth rate" and to the EURL *Lm* Technical guidance document on challenge tests and durability studies for assessing shelf-life of ready to eat foods related to *Listeria monocytogenes*."

To comply with the microbiological criteria for *Lm* set out in Annex I (food categories 1.2 and 1.3) of Regulation (EC) No. 2073/2005, the growth potential (Δ) can be used to:

- Classify a food in:
  - category 1.2 "Ready-to-eat foods able to support the growth of *L. monocytogenes, other than those intended for infants and for special medical purposes* " when $\mathbf{\Delta} > 0.5 \log_{10}$ cfu/g.
  - category 1.3 "Ready-to-eat foods unable to support the growth of *L. monocytogenes*, other than those intended for infants and for special medical purposes" when $\mathbf{\Delta} \leq 0.5 \log_{10}$ cfu/g,
- Quantify the growth of *Lm* 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 main ones being:

- intrinsic factors (e.g. pH, water activity (a_w), organic acids, background microflora, preservatives),
- extrinsic factors (e.g. time-temperature profile, packaging conditions, gas composition),
- physiological state of the inoculated strain(s), and level of contamination.

4.2. Challenge test assessing the maximum growth rate of *Lm*

A challenge test assessing the maximum growth rate is a microbiological laboratory-based study that measures the growth rate of a single *Lm* strain in an artificially contaminated food stored at a constant temperature. The maximum growth rate ($\mathbf{\mu_{max}}$ expressed in natural logarithm) is estimated from the exponential phase of a growth curve of *Lm* obtained at a constant temperature by plotting the natural logarithm of the bacterial population versus the time. The slope of the linear phase is the maximum growth rate (μ_{max}). For further details, refer to the standard EN ISO 20976-1 on "Requirements and guidelines for conducting challenge tests of food and feed products - part 1 : Challenge tests to study growth potential,

lag time and maximum growth rate" and to the EURL *Lm* Technical guidance document on challenge tests and durability studies for assessing shelf-life of ready to eat foods related to *Listeria monocytogenes.*

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

- the growth characteristics of the strain,
- intrinsic factors (e.g. pH, aw, organic acids, background microflora, preservatives),
- extrinsic factors (e.g. temperature, packaging conditions, gas composition).

It is estimated by fitting a primary model to the experimental growth curve and can be used to determine the increase in the bacterial population at any time of the shelf-life of the product and/or used as input in predictive microbiological models.

4.3. 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).

For *Lm*, durability studies are mainly used to determine the concentration of *Lm* at the end of the shelf-life and thus to verify the shelf-life of a food under given storage conditions.

5. ASSESSMENT OF THE LABORATORY EXPERTISE

5.1. 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, food processing, predictive models in microbiology (for laboratories assessing the maximum growth rate) and statistics. The statistical expertise encompasses an understanding of sampling theory, design of experiments and statistical analysis of microbiological data.

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

The laboratory should have knowledge of:

- Regulation (EC) No 2073 / 2005 on microbiological criteria for foodstuffs amended,
- EC/DG SANCO Guidance document on *Listeria monocytogenes* shelf-life studies for ready-to-eat foods under Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs,
- EURL *Lm* Technical guidance document on challenge tests and durability studies for assessing shelf-life of ready to eat foods related to *Listeria monocytogenes*,
- EN ISO 20976-1 on "Requirements and guidelines for conducting challenge tests of food and feed products - part 1: Challenge tests to study growth potential, lag time and maximum growth rate".

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 standardised 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 *Lm* 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,
- to perform metrological quality control tests
- to have successfully participated in proficiency tests.

For using predictive microbiology, the laboratory should have knowledge on the available models and software tools on their validity for the product tested and demonstrate the ability to use it.

5.1.1. Task of the laboratory

The task of the laboratory is to design and conduct the shelf-life study, based on the information from the FBO and to provide at the end of the study a challenge test report or a durability study report.

5.1.2. Review of information from the FBO on the studied product

Product information required for conducting shelf-life studies is stated in the EC/DG-SANCO Guidance document and in the EURL *Lm* Technical guidance document. The FBO should provide to the laboratory relevant product information (see below section 5.1.3).
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 challenge test or a durability study for the specific RTE food and shall, based on the information obtained, design the experimental protocol of the challenge test according to EN ISO 20976-1 and to the EURL *Lm* Technical guidance document. 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.

5.1.3. Information required before initiating a challenge test / a durability study

Before initiating a challenge test/ a durability study, the following relevant information inherent to the studied product should be considered:

- Information on the scope of the study
- Study relevant for a single product / for a product representing a range of products
- Identification of the product
    - Name of the product or identifiable code for new product development
    - Weight of the product
    - Storage temperature
    - List of ingredients
    - Expiry date or assigned date for a new product
    - Photo of the product and label
- Shelf-life of the product
    - Production date
    - Intended microbiological shelf-life
- History of the product
    - New product, new formulation
    - Product commercialised

- Production process
    - Main steps (linked to the inactivation of microorganisms or to possible recontamination)
- Packaging of the product
    - Properties of packaging material (e.g. permeability, …)
    - Packed under air/ under vacuum / under modified atmosphere (gas composition)
- Physico-chemical characteristics of the product
    - Data (number of values, period covered, mean, standard deviation, range) for each factor of concern
    - Factors: pH, a_w or water phase salt (WPS), concentration of preservatives, …
- Microbiological characteristics of the product:
    - Data for Lm (number of values, period covered, prevalence, level of contamination, data exceeding the limit of 100 cfu/g);
    - Data for microorganisms (other than *Lm*) of significance (number of values, period covered, level of contamination):
    - Total microflora, Lactic acid bacteria, … ;
    - Technological microflora (addition of probiotics, starter cultures, ...).
- Characterisation of the cold chain:
    - Storage temperature and duration: at the production, from production to retail, at retail, at consumer level;
    - Destination of the product (national marketplace and/or other EU Member States).

5.2. Drafting of a challenge test report or a durability study report

At the end of the study, the laboratory shall provide a report that outlines the purpose of the challenge test/durability study, the conditions under which the challenge test/durability study has been carried out, the results obtained and a conclusion. For challenge tests, it shall include at least the information listed in the standard EN ISO 20970-1 and the EURL *Lm* Technical guidance document and for durability study in the EURL *Lm* Technical Guidance Document.

The report shall include in an annex, an overview of the data coming from the FBO.

6. ASSESSMENT OF THE TECHNICAL COMPETENCE OF THE LABORATORY

6.1. Challenge tests

The following items, identified in the EN ISO 20976-1 and the EURL *Lm* Technical guidance document on challenge tests and durability studies for assessing shelf-life of ready to eat foods related to *Lm* should be specified in the challenge testing experimental design.

6.1.1. Number of batches

For the growth potential, at minimum three batches have to be tested. It is recommended to 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.
For the maximum growth rate, when less than three batches are tested, the justification (results of the Inter-Batch Physico-Chemical Variability calculator of EN ISO 20976-1) must be given in the report.

6.1.2. Strains

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

- At least 2 strains must be used
- 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
- The origin of the strains shall be given (including the product from which the strain was isolated if known)
- Their growth characteristics, biochemical characteristics and molecular typing characteristics must be documented.

6.1.3. Preparation of the inoculum

The laboratory shall standardise the preparation of the inoculum to avoid introducing as much as possible a bias, when inoculating *Lm* 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 for the first subculture and for the second subculture, at or close to the initial storage temperature of the product
  - For mixed cultures (growth potential), equal concentration of each second subculture shall be mixed.

- Inoculum:
  - The target inoculum concentration shall be obtained by diluting the 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 and on a non selective agar (when a stress protocol is applied to the inoculum).

6.1.4. Inoculation of test units

Based on the collected information provided by the FBO, the laboratory shall choose between the available methods listed in the standard EN ISO 20976-1 and 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 products maintained in their original packaging.

The targeted level of contamination around 100 cfu/g (range between 50-200 cfu/g) shall be respected as well as the volume of inoculation (≤ 1% of the mass of the test unit inoculated).

6.1.5. Storage of test units

This step is of major importance, especially in challenge testing assessing the growth potential. The combination of temperature/duration for each step of the cold chain shall be justified according to table 4 of section 6.2.2.6. of the EURL *Lm* Technical guidance document:

- at manufacturer level: Time/temperature profile supported by information provided by the FBO (95^{th} percentile of FBO's data observation);
- at retail and consumer level: Time/temperature profile based on national data (95^{th} percentile of the observations for the country where the stage of the cold chain is located);
- when no data on the cold chain is available: Time/temperature profile defined as default values (7°C, 7°C and 10°C).

The laboratory shall give evidence that test units are stored under the time /temperature profile defined in the protocol (by use of a temperature control unit).

6.1.6. Physico-chemical measurements of food controls and control units

To characterise the product on which the challenge test is performed and check the representativeness of this product, in comparison to those of common production, the laboratory shall measure, on food controls, relevant physico-chemical parameters, such as:

- pH, a_w or NaCl and moisture content instead of a_w;
- Gas composition;
- Other parameters specific to the product, identified as having an impact on the growth of *Lm*.

The laboratory shall also measure these physico-chemical parameters on control units to ensure that the preparation of test units do not introduce changes of the characteristics of the product.
The laboratory shall specify when the analyses are performed and the number of control samples planned to be checked.
Per batch, at least one sample shall be used at the beginning of the challenge test (for the food control and the control unit) and one sample at the end of the test (control unit).

6.1.7. Microbiological analyses

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

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

The laboratory shall document, for each batch, the number of sampling points (at least five for the growth potential and eight for the maximum growth rate) used and the number of test units analysed per sampling point.
The laboratory shall, because of the targeted contamination level, lower the limit of enumeration to 10 cfu/g.

- To verify that the batch is not naturally contaminated, the laboratory shall carry out the detection of *Lm* on a food control sample at time zero (0), using the reference method EN ISO 11290-1 or an alternative method validated according to ISO 16140-2.

When *Lm* is detected in the studied batch, the laboratory must inform the FBO of the result immediately. For a challenge test assessing the growth potential, because of the mixture inoculation, this test can continue, only if the level of this natural contamination is lower or equal to the artificial one. For a challenge test assessing the maximum growth rate, because of the single inoculation, this test must be stopped.

- To characterise microbiologically the product of concern, the laboratory should enumerate, using food control and control units, the natural microflora relevant for the product: e.g. total microflora (viable mesophilic bacteria), lactic acid bacteria or yeasts, but at least total microflora (viable mesophilic bacteria).

The laboratory shall document: when these analyses are conducted and the number of control units and food control tested. For each batch tested, at least one sample shall be used at the beginning of the challenge test and one sample at the end of the test.

6.1.8. Determination of the growth potential and exploitation of the results

To calculate the growth potential of *Lm* of the studied product, the laboratory shall:

- Determine the concentration of *Lm* (in $\log_{10}$ cfu/g) in the test units, analysed over time (3 at t0 beginning of the test and 4 between t_0 and t_{end}), as described in the standard EN ISO 20976-1 and in the EURL *Lm* Technical guidance document;
- Check for each batch at the time 0, if the standard deviation of the three *Lm* enumerations is ≤ 0.3 $\log_{10}$ cfu/g. If not the case, the challenge test of the batch is inconclusive;
- Use the calculation formula of the growth potential provided in the standard EN ISO 20976-1 and the EURL *Lm* Technical guidance document;
- 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 *Lm* in the studied product.

Any observation that could influence the validity of the data or conclusion must be reported.

6.1.9. Determination of the maximum growth rate and exploitation of the results

To calculate the maximum growth rate of *Lm* in the studied product, the laboratory shall:

- Build the growth curve of a *Lm* strain individually tested (concentration of *Lm* in $\log_{10}$ cfu/g versus time) at one defined temperature;
- Fit a primary model, to all the experimental data points using a microbiological software to estimate the maximum growth rate with its standard error;
- The laboratory shall, from the maximum growth rate obtained for each batch, calculate the average of these values and retained this value with its standard deviation 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 of a secondary model given in the EURL *Lm* Technical guidance document.

6.2. Durability studies

Durability studies are carried out on batches that are likely to be naturally contaminated by *Lm.* These studies differ from challenge tests as samples are not artificially contaminated. Due to high heterogeneity of *Lm* 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 ("prerequisites before initiating a durability study" describe in paragraph 5.1.3) should be considered by the laboratory.
In durability studies, the number of samples above 100 cfu/g can be assessed in terms of frequency and trends.
The following items, identified in the EURL *Lm* Technical guidance document on challenge tests and durability studies for assessing shelf-life of ready to eat foods related to *Lm* should be specified in the durability study protocol.

6.2.1. Food sampling procedure

The laboratory should request from the FBO historical data (prevalence of *Lm*) 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 the sampling procedures for random and targeted sampling, and take the sampling procedure into account in interpretation of the results. For analyses of more than one batch, the distribution in time between batches should be given.

6.2.2. Storage conditions and measurements/analyses of food characteristics

See experimental challenge testing procedures (§6.1.5to § 6.1.7).

6.2.3. Calculation and exploitation of the results

From the samples randomly selected from a batch, the laboratory should calculate the percentage of samples above 100 cfu/g at the end of the shelf-life according to the storage conditions applied and assess the proportion, with its confidence interval, of samples exceeding the limit in the whole batch. For this, the laboratory can use the calculator tool reported in the Technical guidance document (http://www.causascientia.org/math_stat/ProportionCI.html).
All experimental data for *Lm* concentration should be 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 of the result 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 (growth or inactivation) 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 $\log_{10}$ of the highest concentration of the artificially inoculated bacterial population during the challenge test and the $\log_{10}$ of the initial concentration of this bacterial population.

Maximum growth rate: Kinetic parameter to characterise the exponential growth phase, represented by the slope of the curve showing the evolution of the natural logarithm (μ_{max}) or decimal logarithm (V_{max}) of the population as a function of time, under constant temperature.

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): Ratio of the water-vapour pressure in the foodstuff to the vapour pressure of pure water at the same temperature. 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 bacteria. 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 checklist to assess the technical competence of the laboratory performing a challenge test

The following items, identified in the standard EN ISO 20976-1 and the EURL *Lm* Technical guidance document (TGD), should be specified in experimental design for challenge test.

All justification of a deviation from the specifications of the checklist, must be added as a comment (right column of the table below).

	Specifications of ISO 20976-1 and EURL *Lm* TGD	**Yes**	**No**	**Comment**
Batches				
Number of batches to be tested	At least 3 batches	☐	☐	
	One batch for μ_{max}, if inter-batch variability of pH and a_w have no significant impact on the growth rate of *Lm*	☐	☐	
	Use of the inter-batch physico-chemical variability calculator	☐	☐	
Representativeness of the batches	Batches representative of the regular production process variability	☐	☐	
	Batches with the physico-chemical characteristics the most favorable to growth	☐	☐	
	Batches coming from different production days	☐	☐	
Dispatch of batches	Batches tested at three different days	☐	☐	
	Within 2 days after the production day	☐	☐	

	Specifications of ISO 20976-1 and EURL *Lm* TGD	Yes	No	Comment
Strains				
Number of strains	At least 2 strains	☐	☐	
Selection of the strains	Source of the strains known	☐	☐	
	Strains isolated from food matrix, production environment, outbreaks	☐	☐	
	Strains suitable for the product and storage condition	☐	☐	
	With known growth characteristics	☐	☐	
Use	In mixture (growth potential)	☐	☐	
	Individually (maximum growth rate)	☐	☐	
Control of the strains (biochemical, genoserotype, growth)	Control procedures	☐	☐	
	Growth ability tested	☐	☐	
	Cardinal values determined (for μ_{max} determination)	☐	☐	

	Specifications of ISO 20976-1 and EURL *Lm* TGD	Yes	No	Comment
Preparation inoculum				
Preparation of the subcultures	1st subculture in broth in optimal condition until the early stationary phase	☐	☐	
	2nd subculture at or close to the initial T° of the product, until the early stationary phase	☐	☐	
	Enumeration of each 2nd subculture	☐	☐	
Mixture of the subcultures	In equal concentration	☐	☐	
Dilution of the mixture (Δ) or 2nd subculture (μ_{max})	In non-growth promoting diluent (e.g physiological water)	☐	☐	
Use of the inoculum	Immediately	☐	☐	
	Amount of inoculum large enough for all test units inoculation	☐	☐	
Enumeration of the inoculum	On the medium (selective agar) used for the test	☐	☐	
	For stressed inoculum, on selective and non selective agar	☐	☐	

	Specifications of ISO 20976-1 and EURL *Lm* TGD	Yes	No	Comment
Inoculation of the test units				
Initial level of contamination	Targeted level around 100 cfu/g (between 50-200 ufc/g)	☐	☐	
Inoculum volume	Volume of the inoculum ≤ 1% of the mass of the sample inoculated	☐	☐	
Methods of contamination	Depackaged products:			
	• in depth	☐	☐	
	• on the surface	☐	☐	
	• on both	☐	☐	
	For foods with multicomponents:			
	• contamination of the part(s) likely contaminated with *Lm*	☐	☐	
	• contamination at the interface of ingredients	☐	☐	
	Repackaging:			
	• in the initial packaging or with the same material	☐	☐	
	• If other material, properties known and similar to the initial	☐	☐	
	• Same gaseous composition, gas volume and head space	☐	☐	
	Packaged products:	☐	☐	
	• on the surface, or in depth, or both, through a septum	☐	☐	
	• use of a double septum	☐	☐	
Preparation of control units	Addition of a volume of physiological water equivalent to the inoculum volume	☐	☐	
	Preparation of a temperature control unit equipped with a data logger	☐	☐	
	Placed close to the test units during storage	☐	☐	

	Specifications of ISO 20976-1 and EURL *Lm* TGD	Yes	No	Comment
Storage of the test units				
Storage conditions: growth potential	Identification of the stages of the cold chain from manufacturer to consumer	☐	☐	
Time /storage temperatures	Justified from detailed information given by the FBO	☐	☐	
	Justified from data available at national level	☐	☐	
	Use of the values given in table 4 of the EURL Lm Technical Guidance	☐	☐	
Storage temperature: maximum growth rate	At a constant temperature	☐	☐	
Monitoring of the incubators (storage chambers) temperature during the test	Measurements	☐	☐	

	Specifications of ISO 20976-1 and EURL *Lm* TGD	**Yes**	**No**	**Comment**
Minimum number of units required per batch				
For the growth potential	Minimum number of sampling points: 5 points (including t_0 and t_{end})	☐	☐	
	Minimum number of test units per sampling point: 1 test unit except at t_0 (3 test units)	☐	☐	
	If 3 batches are analysed at the same day, only 1 test unit at t_0 per batch	☐	☐	
	Minimum number of control units for microbiological analysis: 2 (t_0 and t_{end})	☐	☐	
	Minimum number of control units for physico-chemical analysis: 2 (t_0 and t_{end})	☐	☐	
	Number of temperature control units: 1 unit	☐	☐	
For the maximum growth rate	Minimum number of sampling points: 8 points (with 5 in the exponential phase)	☐	☐	
	Minimum number of test units per sampling point: 1 unit except at t_0 (3 units)	☐	☐	
	• If 3 batches analysed at the same day, only 1 unit at t_0 per batch	☐	☐	
	Minimum number of control units for microbiological analysis: 2 (t_0 and t_{end})	☐	☐	
	Minimum number of control units for physico-chemical analysis: 2 (t_0 and t_{end})	☐	☐	
	Number of temperature control units: 1 unit	☐	☐	

	Specifications of ISO 20976-1 and EURL *Lm* TGD	Yes	No	Comment
Physico-chemical measurements				
Parameters measured and number of samples analysed per batch	pH measurement	☐	☐	
	• at least 1 control unit at t_0 and at t_{end}	☐	☐	
	• one food control sample at t_0	☐	☐	
	• use of a standardised method or validated method recognised at international level	☐	☐	
	a_w measurement	☐	☐	
	• at least 1 control unit at t_0 and t_{end}	☐	☐	
	• one food control sample at t_0	☐	☐	
	• use of a standardised method or validated method recognised at international level	☐	☐	
	MAP packaging	☐	☐	
	• measurement of the gas composition	☐	☐	
	• at least 1 control unit at t_0 and at t_{iend}	☐	☐	
	• two food control samples at t_0 and t_{end}			
	Other measured parameters	☐	☐	

	Specifications of ISO 20976-1 and EURL *Lm* TGD	Yes	No	Comment
Microbiological Analyses				
Detection Lm	On 1 food control at t_0 according t_0:	☐	☐	
	• reference method EN ISO 11290-1	☐	☐	
	• alternative method validated according to ISO 16140-2	☐	☐	
Enumeration Lm	On 3 test units at t_0 and then on 1 test unit per sampling point according to:	☐	☐	
	• reference method EN ISO 11290-2	☐	☐	
	• alternative method validated according to ISO 16140-2	☐	☐	
	Enumeration limit lowered at 10 cfu/g	☐	☐	
	Verification of the homogeneity of the contamination of *Lm* at t_0 ($\sigma \leq 0.3$)	☐	☐	
Enumeration of the total microflora	On at least 1 control unit at t_0 and t_{end}, according to a national or international standard method	☐	☐	
	On 1 food control sample at t_0	☐	☐	
Enumeration of a specific microflora (recommended)	On at least 1 control unit at t_0 and t_{end}, according to a national or international standard method	☐	☐	
	On 1 food control sample at t_0	☐	☐	

	Specifications of ISO 20976-1 and EURL *Lm* TGD t	Yes	No	Comment
Determination of the growth potential				
Calculation method	Calculation of the Lm concentration in log_{10} at t_0 and at the 4 further sampling points	☐	☐	
	Calculation formula used: Δ = log max –log i	☐	☐	
	Calculation of the standard deviation at t_0	☐	☐	
	Determination of Δ for each batch	☐	☐	
	The highest Δ value is retained	☐	☐	
	Significant increase of Lm if $\Delta > 0.5$ log cfu/g.	☐	☐	
Determination of the maximum 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 maximum growth rate by fitting a primary model to the growth curve	☐	☐	
	Use of a predictive microbiological software	☐	☐	
	Determination of the standard error for each estimated μ_{max} (1 per batch)	☐	☐	
	The growth rate retained is the mean of the 3 μ_{max} estimated values with its standard deviation	☐	☐	
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 ISO 20976-1 and EURL *Lm* TGD t	Yes	No	Comment
Test report				
Purpose of the study and Type of challenge-test	Validation of the food shelf-life, scope (single / group of products)	☐	☐	
Identification of the food	Name of the product or identifiable code for new product development (NPD)	☐	☐	
	Description (composition, structure, packaging, photo)	☐	☐	
	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 per batch	☐	☐	
	Strains used	☐	☐	
	Preparation of the inoculum	☐	☐	
	Inoculum concentration	☐	☐	
	Test units preparation: Mass or volume of the test units, volume of inoculum introduced per test unit, ratio met, packaging characteristics	☐	☐	
	Inoculation method	☐	☐	
	Date(s) of inoculation	☐	☐	
	Level of contamination targeted	☐	☐	
	Duration of the test, sampling times	☐	☐	
	Storage temperature/duration and justification	☐	☐	
	Lm enumeration and Lm detection methods used	☐	☐	
	Limit of Lm enumeration	☐	☐	
	Physico-chemical values at t_0 and at t_{end}	☐	☐	
	Methods used	☐	☐	
	Gas atmosphere composition	☐	☐	
	Temperature of the control unit	☐	☐	
	Concentration of total and associated microflora at t_0 and t_{end}	☐	☐	
	Enumeration methods for total and associated flora	☐	☐	

	Concentration of *Lm* in the test units at each sampling point Growth potential per batch/ retained for product under the storage conditions applied.	☐ ☐	
	Growth rate per batch / retained for the product tested	☐ ☐	
Conclusion	State the purpose of the study	☐ ☐	