EURL Lm
European Union Reference Laboratory for *Listeria monocytogenes*
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EURL *Lm* TECHNICAL GUIDANCE DOCUMENT

on challenge tests and durability studies for assessing shelf-life of ready-to-eat foods related to *Listeria monocytogenes*

Version 4 of 1 July 2021

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Foreword

This document is the fourth version of the European Union Reference Laboratory for *Listeria monocytogenes* (EURL *Lm)* Technical Guidance Document (TGD) on challenge tests and durability studies for assessing shelf-life of ready-to-eat foods for *Listeria monocytogenes*. It replaces the third version of 6 June 2014 – Amendment 1 of 21 February 2019.

The first version of this TGD (2008) was prepared at the request of the Directorate General Health & Consumers (DG SANCO) of the European Commission (EC) in response to the needs expressed by EU Member States to have a document providing both detailed and practical information on how to conduct shelf-life studies on *Listeria monocytogenes* (*Lm*) in ready-to-eat (RTE) foods to ensure compliance to the microbiological criteria set out in Article 3.2 of Regulation (EC) No 2073/2005.

The purpose of this revision is to ensure consistency between the EURL *Lm* Technical Guidance Document and 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 the growth potential, lag time and maximum growth rate" published in 2019.

The standard specifies the protocols for carrying out challenge tests for growth studies for any bacteria and yeasts that do not form mycelium, whereas the EURL *Lm* Technical Guidance Document covers the technical aspects specific to *Lm* in RTE foods, which are not addressed in the standard. Therefore, the EURL *Lm* Technical Guidance Document should now be read in conjunction with the standard and be considered as a supplementary document to the standard EN ISO 20976-1.

The revision of this TGD also includes the experience gained over the years in carrying out challenge tests.

This document remains complementary to the EC/DG SANCO document, 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”.

The fourth version of the TGD was prepared by EURL *Lm* together with a working group of six NRLs *Lm* and was approved by the Standing Committee on Plants, Animals, Food and Feed on 1 July 2021.

1 Introduction

1.1 *Listeria monocytogenes*

The genus *Listeria* currently consists of 20 species including *Listeria monocytogenes* a pathogenic bacterium that may cause a disease called listeriosis that may affect humans and a large number of animal species.

Microscopically *Lm* appears as a small gram-positive rod (0.5-2 μm x 0.5 μm), occurring singly or arranged in short chains, motile at 20-25°C and non-spore-forming. It is aerobic and facultatively anaerobic, catalase-positive except for a few rare strains, oxidase negative and esculin positive. *Listeria* ferments many carbohydrates without producing gas. Strains of *Lm* are D-xylose negative and produce lecithinase. They are generally β-haemolytic and L-rhamnose positive.

Lm is genetically diverse: The strains are classified into four evolutionary lineages (I-IV), 13 serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7) based on conventional serotyping (somatic and flagellar antigens) and 4 major molecular serogroups (IIa., IIb, IIc and IVb) based on PCR tests.

Historically serotype 4b (serogroup IV b) was the most prevalent serotype in human clinical cases and was less frequently recovered from foods. However, over the last decade, serotype1/2a (serogroup IIa) was the most prevalent serotype in food and environmental samples, and has been frequently linked to human disease, causing notable outbreaks in Europe and North America.

Recently, outbreak investigations have shifted from using pulsed field electrophoresis (PFGE) considered previously as the "gold standard" for bacterial typing, to Whole Genome Sequencing (WGS) typing, which has a higher discriminating power compared to PFGE (Gillesberg Lassen et al., 2016).

Large typing studies carried out by Multi Locus Sequence Typing (MLST) reveal that the *Lm* population is largely clonal. Most strains are gathered into a few important Clonal Complexes (CCs) that are defined as groups of isolates exhibiting sequence types (STs). Today, CCs and STs are systematically used to classify the strain populations.

Hypervirulent and hypovirulent CCs were distinguished by combining epidemiological, clinical and experimental approaches (Maury et al., 2016). The strains of CC1, CC2, CC4, CC6 (Lineage I) accounted for a majority of listeriosis outbreaks and sporadic cases in humans and animals. Other CCs such as CC9, CC121 (Lineage II) are more often isolated in highly immuno-compromised patients. These CCs are overrepresented in food, prevalent in all food sectors (Felix et al., 2018) and able to persist over many years in different food processing environments. However, WGS is expected to give deeper and more nuanced knowledge in this area in the future.

Lm is a ubiquitous, telluric bacterium, widely distributed in the environment. It is a psychrotrophic bacterium able to grow at refrigeration temperatures (Table 1).

Table 1: Growth /survival characteristics of *L. monocytogenes* (strain-specific) in broth medium

	Min. (lower growth limit)	**Growth Optimum** (fastest growth)	**Max.** (upper growth limit)	**Survival** (but no growth)
Temperature (°C)	-2	30 - 37	45	-18
pH	4.0 - 4.3	7.0	9.6	3.3 – 4.2
a_w	0.92 (0.90 with glycerol)	0.99	/	<0.90
NaCl content			12	≥20
Gas atmosphere	Facultative anaerobic and microaerophilic (able to grow in presence / absence of O2. (e.g. under vacuum or modified gas atmosphere)			

Sources: *Lm* growth characteristics refer to Anses datasheet on biological hazards "*Listeria monocytogenes*", 2020 and *Lm* survival characteristics refer to EC/DG SANCO 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".

L. monocytogenes is generally transmitted when food is harvested, processed, prepared, packed, transported or stored in environments contaminated with *Lm*. Most ready-to-eat foods are susceptible to *Lm* contamination, but only those in which *Lm* can survive and/or grow are potential causes of listeriosis. Transmission through food is by far the most important (99% of cases) transmission pathway to human. After eating food contaminated with *Lm*, people may develop listeriosis.

Listeriosis occurs in both invasive (maternal-neonatal and non-maternal-neonatal) and non-invasive (gastroenteric) forms (Table 2).

Table 2. Types of listeriosis and symptoms

Type of listeriosis	**Incubation time**	**Main symptoms**	**Health impact**
Maternal-neonatal form	17 to 67 days median: 28 days	- Flu-like syndrome (fever...) - Spontaneous abortion - Death in utero, prematurity - Neonatal infection	20% to 30% lethality in new-borns
Non-maternal-neonatal forms	Bacteraemic form: 1 to 12 days median: 2 days Neuro-meningeal form: 2 to 14 days median: 9 days	- Septicaemia - Meningitis, meningo /rhombencephalitis,	Neurological sequelae Lethality from 20% to 30%
Gastroenteric form	6 hours to 4 days median: 24 hours	- Fever - Nausea, vomiting, diarrhea	

Sources: Anses datasheet on biological hazards "*Listeria monocytogenes*", 2020

Invasive listeriosis is the more severe form of the disease and affects particularly certain high-risk groups of the population. These include pregnant women and their newborns, the elderly, and people with weakened immune systems.

1.2 Legislative background

Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs lays down the rules to which the Food Business Operator (FBO) must comply and specifies the microbiological criteria for certain microorganisms.

Annex I of this Regulation sets out the microbiological food safety criteria applicable for *Lm* in RTE foods (criteria 1.1 to 1.3), with criterion 1.1 specifically targeting RTE intended for infants and for special medical purposes and the other two criteria (1.2 and 1.3) targeting all other types of RTE foods. A quantitative limit of 100 cfu/g is set in Regulation 2073/2005 for criterion 1.3 (RTE foods not able to support the growth of *Lm)* and for criterion 1.2 (RTE foods able to support the growth of *Lm*) when the manufacturer is able to demonstrate, to the satisfaction of the competent authority, that its product will not exceed the limit 100 cfu/g throughout the shelf-life.
Article 3, paragraph 2 and annex II of this Regulation specifies that FBOs shall conduct, as necessary, studies to evaluate the growth of *Lm*, that may be present in the product, during the shelf-life under reasonably foreseeable storage conditions.

1.3 EU guidance documents

The EC/DG SANCO document, entitled “Guidance document on *Lm* shelf-life studies for ready-to-eat foods, under Regulation (EC) No. 2073/2005 on microbiological criteria for foodstuffs”, is directed at FBOs who produce ready-to-eat foods and may be used by Competent Authorities (CAs) to verify the correct implementation of shelf-life studies by FBOs.

The aim of this document is to guide FBOs producing RTE foods in identifying the *Lm* risk in their products, and on how to proceed to demonstrate, to the satisfaction of the Competent Authority, that their products will not exceed the quantitative criterion (1.2a and 1.3 of Regulation (EC) No 2073/2005) throughout the shelf-life. A decision tree showing a schematic approach for the steps of shelf-life studies provides FBOs an indication of when additional specific studies are needed to investigate the growth of *Lm* in the product.

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" is intended to be used by CAs and NRLs if mandated by their CAs. The aim of this guidance document is to set up a harmonized approach on how laboratories should conduct challenge tests and how to evaluate laboratories competence in conducting shelf-life studies related to *Lm*. It was prepared by the EURL *Lm* in collaboration with representatives of National Reference Laboratories for *Lm* (NRLs *Lm*) and CA.

2 Scope

- The EURL *Lm* Technical Guidance Document (TGD) should be read in conjunction with 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 the growth potential, lag time and maximum growth rate".

  The standard EN ISO 20976-1 specifies the protocol for conducting challenge tests for any bacteria or yeasts that do not form mycelium and the EURL *Lm* Technical Guidance Document, as a complementary document, gives specifications concerning *Lm.* It contains specific sections to be considered for assessing the shelf-life of ready to eat foods related to *Lm*.

  This document provides guidance on how to perform studies set out in annex II of Regulation (EC) 2073/2005 that can be implemented for assessing the shelf-life of RTE foods with regards to *Lm*:

  (1) Challenge tests (challenge test to determine the growth potential and challenge test to determine the maximum growth rate);

  (2) Durability studies.

- Challenge tests aim to validate the shelf-life of a food product under given storage conditions by providing information on the behaviour of *Lm* (growth, survival, or decrease) when artificially inoculated. Challenge tests shall consider the variability of the batches, of the food samples and of the strains. The level of contamination, the heterogeneity of the contamination and the physiological state of the bacteria is difficult to mimic in a challenge test. Thus, the contamination method cannot always fully mimic the natural contamination.

- Durability studies aim to verify the shelf-life of a food product under given storage conditions. Durability studies evaluate the growth or survival of *Lm* that may be naturally present in a food during their shelf-life, under reasonably foreseeable conditions of distribution, storage and use.

  Even if durability studies may be considered more realistic than a challenge test, as the contamination is naturally occurring, their use in validation is limited. Because of the low *Lm* prevalence, the low *Lm* contamination level and its heterogeneous distribution in the food, it is not recommended to implement durability studies to validate a shelf-life related to *Lm*. Its use is more appropriate for shelf-life verification.

- The choice of the study(ies) to be implemented should be done by the FBO, if necessary with the collaboration of the competent laboratory, that will conduct it. The choice should be based on the information to be obtained, as illustrated in Figure 1.

Figure 1 presents the possible types of studies for determining the growth of *Lm* in RTE foods and the results obtained by each one.

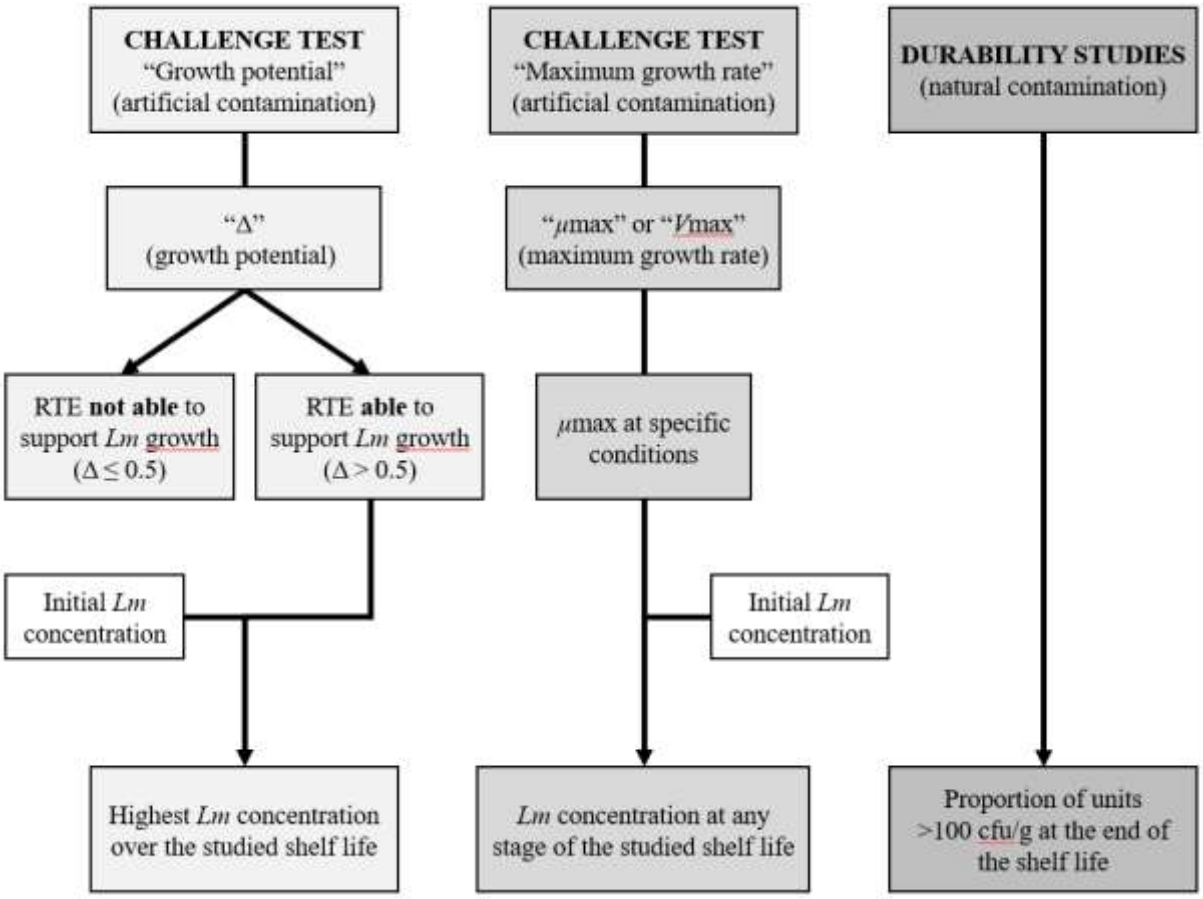

Figure 1. Results obtained by each type of study

A table outlining the benefits and limitations of each type of study is presented in annex 10.1.

The TGD is basically intended for laboratories conducting challenge tests and durability studies on *Lm* in RTE foods, on behalf of the FBOs.

The TGD mainly applies to pre-packed[1] RTE foods intended for consumer and for mass caterers[2], as defined in EU Regulation 1169/2011, for which a "use by date" has to be determined.

[1]prepacked food' means any single item for presentation as such to the final consumer and to mass caterers, consisting of a food and the packaging into which it was put before being offered for sale, whether such packaging encloses the food completely or only partially, but in any event in such a way that the contents cannot be altered without opening or changing the packaging; 'prepacked food' does not cover foods packed on the sales premises at the consumer's request or prepacked for direct sale.

[2]mass caterer' means any establishment (including a vehicle or a fixed or mobile stall), such as restaurants, canteens, schools, hospitals and catering enterprises in which, in the course of a business, food is prepared to be ready for consumption by the final consumer.

Challenge tests for pre-packed RTE foods should be conducted using the product in its final packaged format, taking into account the reasonably foreseeable conditions of distribution, storage and use. The shelf-life is established for the product as it is packed when sold.

For unpacked products, additional factors, such as hygrometry, have to be considered for the storage of the product under reasonably foreseeable storage conditions; it is thus necessary to adapt the experimental protocol to this type of products.

For products which are intended to be displayed in bulk (i.e. large blocks of cheese, pieces of ham or tubs of deli-salads), the tests should be conducted using the typical packaging which is expected to be supplied to caterers or consumers (e.g. ham may be overwrapped with packaging film, salads may be filled into plastic pots).

3 Normative references

The latest edition of the referenced document (including any amendment) applies:

- 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;
- 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 the food chain - Horizontal method for the detection and enumeration of *Listeria monocytogenes* and of *Listeria* spp. - Part 2: Enumeration method;
- 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;
- ISO/NP 23961, Microbiology of the food chain - Determination and use of cardinal values.

4 Definitions

4.1 Ready-to-eat (RTE) food

A ready-to-eat food is a 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 the micro-organisms of concern. (Regulation (EC) No 2073/2005).

Ready-to-eat foods are likely to be contaminated but the level and frequency of contamination is variable and generally low. Foods in which *Lm* can survive and/or grow are potential causes of listeriosis when the storage (temperature/time) or preparation instructions described by the FBO on the packaging are not followed.

RTE food associated with human listeriosis mainly belong to 'meat and meat products', 'fish and fish products', and 'milk products' categories. Outbreaks continue to occur across the globe, associated with many previously unreported food vehicles, including food of plant derived origin such as fresh and minimally processed fruits (cantaloupe, caramel apples) and vegetables, sprouts. Outbreaks are also linked to changing consumer habits such as the use of frozen foods destined for cooking but used thawed (frozen corn).

4.2 Shelf-life of RTE foods

The shelf-life of a food is defined as a period of time for which a product remains safe and meets its quality specifications under reasonably foreseeable conditions of storage, distribution and use.

According to EC Regulation 2073/2005, this shelf-life correspond to the period preceding:

- the “use by date” i.e. the last day the food product must be used. In this case, shelf-life relates to food safety;

or

- the “date of minimum durability” or “best before date” i.e. the date until which the food retains its specific properties when properly stored. This relates mainly to food quality (appearance, odour, texture, flavour, etc.).

The microbial shelf-life of a food corresponds to the period of time during which the food remains within predefined quantitative microbiological limits. It begins from the time the food is produced and/or packed. The microbial shelf-life of a food is a food safety control measure that has to be validated by studies laid down in Annex II of Regulation (EC) No 2073/2005.

5 Role of the FBO and laboratory

It is the FBO's responsibility to conduct such shelf-life studies, to establish the compliance with the microbiological criteria throughout the shelf-life of a product (annex 10.2). Challenge tests may be carried out as a part of a shelf-life study. The FBO is responsible for setting the shelf-life under defined conditions, which should take into account reasonably foreseeable conditions during transportation, storage at manufacturer, at retail and at consumer levels. The role of the FBO is to establish a food safety management system, provide relevant data on the product characteristics, production process, storage conditions, considering the inherent variability linked to the product, processing and storage conditions.

The task of the laboratory is to design and conduct the challenge test or durability study based on the information provided by the FBO. The laboratory should have the required expertise or else have access to relevant knowledge in food microbiology, food sciences, food processing and statistics. The statistical expertise encompasses an understanding of sampling theory, design of experiments and statistical analysis of microbiological data.

The analyses in a challenge test shall be conducted under a quality assurance system. It is recommended to use a laboratory with accreditation to EN ISO/IEC 17025 for the analytical methods used in the challenge test and at a minimum the methods for the detection and enumeration of *Lm*.

For non-accredited laboratories, the minimum quality assurance level expected is that they have documented good laboratory practices, perform own metrological quality control tests and have successfully participated in proficiency tests.

The role of the laboratory is to present the results of the challenge test or durability study, including the conclusion on the behaviour of *Lm* in the tested product, in a report (challenge test report or durability study report) which can be used as part of a shelf-life study by the FBO.

Finally, it is the responsibility of the FBO to interpret the results and conclusion of the laboratory report. This interpretation should be recorded in a shelf-life study report, which should also include additional information (e.g. the initial contamination level of the product directly after production, information about the physico-chemical characteristics of the products, information about the storage time-temperature profile, information about the production process, etc.).

The responsibility of the FBO is to make the shelf-life study report of a product available to CAs upon request to allow for evaluation.

6 Challenge test

A challenge test is a laboratory-based study used to evaluate the microbiological safety of a product. It will assess whether a product is able or not to support the growth of *Lm*.

In foods, many factors or combination of factors may influence the growth of *Lm*. These factors are divided into intrinsic and extrinsic parameters (annex 10.3). Intrinsic parameters (related to the food itself) include pH, water activity (a_w), NaCl, moisture content, background microflora, nutritional content and structure of the food, preservative content. Extrinsic factors (related to the storage environment of the food) include gas atmosphere, relative humidity, packaging and time-temperature storage conditions.

Some of the factors that influence the growth of *Lm*, may vary within a batch (intra-batch variability) or between batches (inter-batch variability) and these variabilities have to be assessed before starting a challenge test.

6.1 Prerequisites before initiating a challenge test

Before starting a challenge test, the FBO should be able to provide the laboratory with relevant information inherent to the studied product. The goal of this first step is multiple and allows the following:

- Identify the factors that have an impact on the growth of *Lm*, and give priority to measurements on these factors;
- Assess the sources of variability of the factors that characterise the product and production process;
- Demonstrate that products analysed during the challenge tests are representative of the production.

The relevant product information required by the laboratory is the following:

- Description of product (commercial name of the product, weight, …), new formulation, new product or a product with a production history;
- Processing conditions (at least the relevant ones in the production process: For instance thermal treatment, drying, smoking, ripening, slicing, mincing, freezing, thawing, salt curing, packaging, etc…);
- Composition of the product (labelled on the product);
- Product characteristics including the variability between and within batches of the product. It is also important to note, for certain categories of food, if the values of certain characteristics change during the shelf-life (e.g pH values in fermented products, cheeses; or aw values in dry ham, hard cheeses);

- Packaging condition of the end-product (including a photo of the product);
- Storage conditions during the shelf-life (taking into account reasonably foreseeable conditions during transportation, storage at manufacturer, at retail and at consumer levels);
- Shelf-life, recommended (instructions on the packaging) and reasonably foreseeable conditions of use of the product.

A flow diagram describing schematically the different steps, from FBO historical data to test in the laboratory, is given in annex 10.4.

To characterise a product, it is recommended to estimate intra-batch variability on a minimum of five values and the inter-batch variability on a minimum of three different batches analysed on a period reflecting the possible variability. This minimum number of data could be considered as a starting point to characterise the variability of the factors that impact the growth of *Lm*.

Examples on how to assess, according to the available amount of historical data, whether the characteristics of the studied batches are representative of the variability observed under normal processing conditions, are given below.

- Example 1 = Ideal situation where the FBO has numerous historical data (product manufactured for many years)

Gathering data recorded by the FBO at manufacturing level over the years, gives a good information on the distribution of pH measurements on the commercialized product (Figure 2).

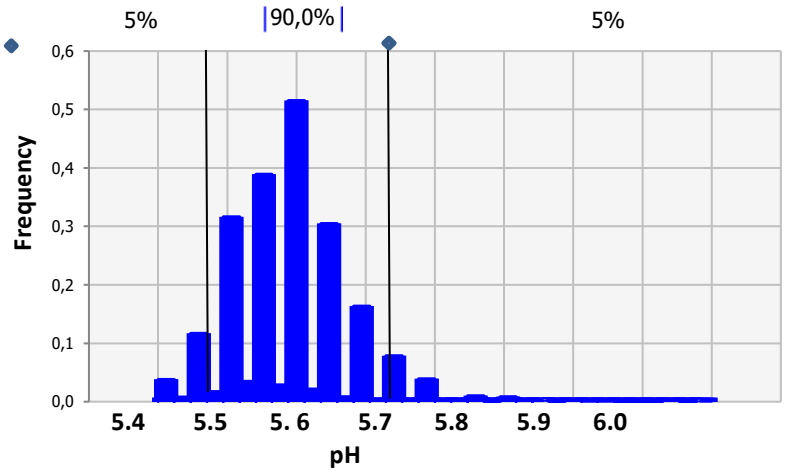

Figure 2. Histogram of pH values of a RTE food from historical data

Based on this distribution of pH measurements, the food controls of the challenge test can be considered representative of the variability of the production process, if their pH values are within the 90% range of the historical data.

- Example 2 = A situation where the FBO has limited historical data.

The FBO has characterised three batches of a food product with only one measurement per batch (Table 3).

Table 3. Table of physico-chemical and microbiological characteristics of a RTE food

	PH	a_w	Preservative	Total microflora
Batch 1	5.5	0.98	1.0%	3.10^4
Batch 2	5.1	0.98	1.5%	9.10^4
Batch 3	5.2	0.98	1.2%	5.10^4

In this case, it is not possible to assess whether the tested batches will be representative of the production process. It is not reliable to characterise a product with such limited data. There should be at least 5 values for each factor per batch.

6.2 Challenge test assessing the growth potential

6.2.1 Introduction

A microbiological challenge test assessing a growth potential (Δ) is a laboratory-based study that measures the growth of *Lm* in artificially contaminated food stored under foreseeable conditions at manufacturer, at retail and at consumer levels. A microbiological challenge test has to reflect the foreseeable conditions that might be expected to occur throughout the cold chain, including storage conditions between production and consumption. The test period starts the day of contamination and finishes at the end of the shelf-life.

The growth potential (Δ) is the difference between the highest observed *Lm* concentration in $\log_{10}$ cfu/g during the test and the initial *Lm* concentration in $\log_{10}$ cfu/g at the beginning of the test.

Growth potential (Δ) = (highest observed *Lm* concentration) - (initial *Lm* concentration)

In the frame of the implementation 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 *Lm* 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 *Lm* other than those intended for infants and for special medical purposes" (category 1.3),
- Quantify the growth of *Lm* in a food of category 1.2 according to defined reasonably foreseeable conditions between production and consumption.

In a challenge test assessing growth potential, stresses or adaptation could be applied to the cell suspensions in order to mimic the physiological state of the bacteria which are most likely to contaminate the product.

Few examples to illustrate this:

- *Lm* strains which are likely to contaminate a packaged cooked sliced meat would come from a chilled manufacturing environment and so the strains should be adapted to grow in chilled conditions;

- Other processed foods, e.g. high pressure processed (HPP) pate, may not be intended to achieve a complete inactivation of any *Lm* present and so if the aim is to determine the shelf-life after the HPP treatment, then the *Lm* strains used could be exposed to HPP treatment as part of the inoculum preparation.

The drawback of this test is the lack of flexibility in the interpretation: the results are only valid for the product tested under the specified conditions, so that new experiments have to be performed each time there is a change (e.g. use of different time-temperature profiles, change of ingredients or recipe).

6.2.2 Protocol of a challenge test to assess growth potential

6.2.2.1 Choice of batches

As a rule, at least 3 batches have to be tested for determining the microbiological shelf-life of a RTE food, in order to be able to capture the inter batch variability.

The batches should be sent to the laboratory as soon as possible after the production (i.e. the day of production or the day after) to avoid any changes in the food characteristics between the day of production and the day of inoculation (see also paragraph 6.2.2.4).

To account for variation of the production process and of the product, it is recommended to analyse three batches coming out from different production days. It means practically to conduct three different challenge tests.

Note: Where different batches are produced on the same day, and where between batches variability is covered over a day (to be justified), then performing the analyses of the 3 batches produced on the same day can be accepted.

6.2.2.2 Choice of strains

To account for variation in growth and survival among strains of *Lm*, a challenge test should be performed with a mixture of *Lm* strains. The same *Lm* strains are to be used for the three batches.

Growth of *Lm* strains varies depending on the food and storage conditions studied. To help the laboratory to find and choose *Lm* strains, EURL *Lm* has constituted a set of *Lm* strains isolated from different origins (meat, fish, milk products and environment). These strains have been characterised for their growth abilities (μ_{max} have been determined in harsh pH, a_w and temperature conditions (annex 10.5). Examples of how to select a EURL *Lm* strain are also given in this annex.

The EURL *Lm* set of strains is available to the NRLs and can then be provided by the NRL to laboratories across the country on request.

Lm strains should be stored in the laboratory by a method which minimises or eliminates mutations which may affect their growth or survival characteristics. Growth, biochemical and molecular typing characteristics should be checked on a regular basis by the laboratory in charge of the challenge test execution.

6.2.2.3 Preparation of the inoculum

Standardization of the preparation of the inoculum is particularly important to be able to inoculate the RTE food at the expected concentration of 100 cfu/g or ml (range 50 to 200 cfu/g). (an example of calculation is explained in annex 10.6).

In order to be ready to inoculate the batch on the day of arrival at the laboratory, the subcultures for the inoculum preparation should be carried out in advance. The total volume of the prepared inoculum suspension should be large enough to be able to inoculate all the test units.

6.2.2.4 Inoculation of the test units

The inoculation of the test units is a critical step in the performance of a challenge test. It should be performed as soon as possible after the production of the batch, which means within 2 days. If there is a delay in transportation of the samples to the laboratory, or if the product has a long shelf-life (e.g. more than 6 weeks), then the delay between the production day and the start of the challenge test could be longer than 2 days. When this occurs, it should be demonstrated that the time between production and inoculation does not affect the structure, physico-chemical characteristics and microbiological flora of the product.

In a challenge test, the *Lm* inoculation process should ensure it is homogeneously distributed in the food, even if in reality this may not be the case. Thus, the standard deviation of *Lm* enumeration obtained at day 0 (the day of inoculation) from the three replicates should be calculated and be lower than 0.3 log cfu/g. If this standard deviation is higher, then the challenge test for the tested batch is not acceptable, and a new challenge test on another batch needs to be performed.

Different inoculation techniques can be used to artificially contaminate RTE foods:

- Either the food is removed from its packaging, inoculated and then repacked under similar gas conditions as an unopened pack (consumer pack). In this case, the laboratory will use specific trays and films with similar barrier properties compared to the original packaging. The RTE food can be inoculated:
  - in depth: for food considered to be homogeneous (e.g. ground food) or food prepared by mixing several materials (e.g. mixed salad),
  - at surface: to mimic contamination of a specific part during process (e.g. products contaminated during slicing).

    For products having multiple components or layers, one or few relevant components regarding *Lm* contamination and/or the interfaces between components should be inoculated (for example: sandwich).
- Or the food is maintained in its packaging by inoculating through a septum which is immediately covered by a second septum to maintain exact gas conditions.

Some examples of different contamination techniques are detailed in annex 10.7. Other techniques can be used if it can be demonstrated that the moisture content is not changed and will not affect intrinsic properties of the food (for example: dipping).

Whatever the contamination technique used, it is desirable to test it, before the inoculation with *Lm*, by using a diluted dye to visualise the dispersion of the inoculated volume.

For depacked-repacked products under modified atmosphere, care should be taken to ensure that headspace volume and gas composition of the challenge test samples mimic the commercial food product as closely as possible.

Contamination level

The contamination level targeted is around 100 cfu/g (range between 50 – 200 cfu/g).
This level of contamination reduces the effect of measurement uncertainty associated to low numbers. In specific cases, such as inoculation of fermented products, a higher level of *Lm* contamination can be used to be able to easily enumerate and follow the *Lm* growth among other strains on the selective agar.

6.2.2.5 Number of units - Number of sampling points

Refer to EN ISO 20976-1 for the parts related to:

- **The number of units to be prepared** (number of test units to be inoculated, number of control units and food control samples);
- **The number of sampling points.**

See also annex 10.8 of the present document.

6.2.2.6 Storage conditions

The storage conditions applied during challenge testing (incubation of the test units) have to comply with the conditions at which the product is most likely to be subjected in normal use, until the end of the shelf-life. This should include the foreseeable temperature range along the cold chain: from the manufacture to retail, storage at retail and storage at consumer.

Temperature during shelf-life is a critical part of a challenge test assessing the growth potential (annex 10.9). It is the responsibility of the FBO to ensure that the storage conditions used are realistic, taking into account that storage temperatures labelled on the packaging could not always be maintained throughout the cold chain (from production to consumption). If an inappropriate storage temperature (lower temperature than the usually encountered) is used during the challenge test, there may be an underestimation of *Lm* growth and an overestimation of the safe shelf-life length.

- Storage temperature and duration

The temperature(s) used to determine shelf-life of the product has (have) to be properly justified and documented by the FBO.

- For the first stage of the cold chain (from manufacture until the arrival to the display cabinet), when the FBO has its own data, the use of this information is preferred. In this case, use the 95th percentile of the FBO's data observation. If no data is available, use the default temperature of Table 4.

- For the second stage (at retail: display cabinet) and the third stage (consumer storage) of the cold chain when information is available, the use of national data, where the stage of the cold chain is located, is preferred. In this case, use the 95th percentile of the data observation. If no data is available, use the default temperature of Table 4.

Table 4. Flow diagram of storage conditions throughout the cold chain

Stage of cold chain	Storage (incubation) temperature			Storage (incubation) duration			
						Shelf-life (SL) ≤ 21 days	Shelf-life (SL) > 21 days
At manufacturer level	Temperature justified by detailed information*	Or if not known	7°C	Duration justified by detailed information	Or if not known	1/3 SL	7 days
At retail level	Temperature justified by detailed information**	Or if not known	7°C	Duration justified by detailed information	Or if not known	1/3 SL	1/2 (SL-7)
At consumer level	Temperature justified by detailed information**	Or if not known	10°C	Duration justified by detailed information	Or if not known	1/3 SL	1/2 (SL-7)

* Temperature justified by detailed information: the 95th percentile of the FBO's data observation

** Temperature justified by detailed information: the 95th percentile of the observations for the country where the stage of the cold chain is located.

6.2.2.7 Measurement of the physico-chemical parameters

The physico-chemical characteristics of the product shall be known. The basis is the pH and a_w. Instead of a_w, NaCl content and [moisture or dry matter content] can be used as well, for products where NaCl is the factor that monitor the water activity. From these data, firstly calculate the water phase salt content WPS (in g/100ml):

$$WPS = \frac{salt\ content\ (in\ g\ per\ 100g)}{moisture\ content\ (in\ ml\ per\ 100g)\ x\ salt\ content\ (in\ g\ per\ 100g)}\ x\ 100$$

and then estimate the a_w with the following equation:

$$a_w = 1 - 0.0052471\ x\ WPS - 0.00012206\ x\ WPS^2$$

This formula (Resnik, Chirife 1988) (Gimenez, Dalgaard, 2004) is based on the salt content but other components such as sugar content can modify the values of a_w, and in that case this formula cannot be used.

A calculator available in the FSSP (Food Safety and Spoilage Predictor) software (free online: http://fssp.food.dtu.dk/), can be used for calculating the water phase content knowing the % of dry matter, the % of NaCl in the product, and to calculate the water activity (annex 10.10).

In addition, other factors (e.g. organic acids), shall be measured, as far as they are relevant to control the growth of *Lm* in the product (examples in annex 10.11).

To measure the physico-chemical properties of the product, standardised methods (e.g. ISO, EN or national) are preferred. These measurements shall be compared to data coming from the regular production of the food in order to demonstrate that the batches used in the challenge test are representative for the normal production process and that, preferably, the batch that is used in the challenge test represents a worst-case scenario.

When measuring the physico-chemical properties of a product, a representative part of the product shall be tested, taking into account where *Lm* is expected to be present in the product. Below are some examples to illustrate this:

- Multi component meal (composite products):
  The worst case component should be determined and be used for further testing when the various components are separated within the food package and do not interact with each other. When the separate components do have the possibility to interact with each other, the growth of *Lm* will be influenced by the micro-environment, and this shall be taken into account.

- Smoked fish:
  Lm is likely to be present on the surface of the product rather than on the inside. Therefore the chemical characteristics of the surface of the product are of importance specifically. These characteristics might be different from the interior of the product.

When the measurement of these physico-chemical parameters is outsourced, then it is necessary to provide for at least 2 additional control units specifically dedicated for this purpose.

- Gas atmosphere

For repacked test units conditioned under vacuum, it is important to be sure of the performance of the vacuum machine to obtain the same initial vacuum condition.

For repacked test units, initially conditioned under modified atmosphere, it is important to use the same modified atmosphere packaging (MAP) conditions: the gas composition, the gas/product ratio and the gas permeability of the packaging material (similar barrier properties).

For test units packed under modified atmosphere and inoculated through a septum in the original packaging, it is important to verify the tightness of the packaging throughout the duration of the challenge test. Therefore, the gas composition should be measured with a headspace gas analyser to ensure that no leakage in the packaging occurs during the storage period.

These analyses should be performed on a food control sample at the beginning of the test (t_0) and at the end of the test (t_{end}) on a food control sample (without septum) and on a control unit (with septum) to check the tightness of the packaging all over the test period. (annex 10.12).

- Temperature

The measurement of the storage temperatures of the test units shall be recorded throughout the test using a thermal data logger in a dedicated control test unit placed in the same incubator and as close as possible to the remaining test units.

6.2.2.8 Microbiological analyses

Initial suspensions are prepared, if possible, by using the entire contaminated test unit, in order to account for the heterogeneity of the product (annex 10.13).

- *Lm* detection and enumeration methods

According to Annex I of Regulation No. 2073/2005, the reference methods for the detection and enumeration of *L. monocytogenes* are respectively EN ISO 11290-1 and EN ISO 11290-2. As the test units are artificially contaminated with *Lm*, it is not necessary to proceed with the confirmation step when the enumeration is performed.

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 16140-2 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.

From *Lm* detection analyses, when *Lm* is detected in the food control at time 0, it is possible to continue to perform the challenge test assessing the growth potential on the studied batch (because of the multiple bacterial strains inoculation), only if the level of the *Lm* natural contamination is lower or equal to the level of inoculation, which should be in the range [50-200 cfu/g].

For *Lm* enumeration analyses, since the targeted contamination level is about 100 cfu/g (range between 50-200 cfu/g), it is recommended to:

- Lower the limit of enumeration at 10 cfu/g, according to EN ISO 11290-2 by:
  - using 1 ml of the initial suspension spread onto 3 plates of ∅ 90 mm, or spread onto 1 large plate of ∅140 mm,
  - or, for validated alternative methods, pour-plated 1ml into 1 plate of ∅ 90 mm.
- Apply a method with lower detection level for enumeration than the EN ISO 11290-2, provided that it is validated and found suitable for enumeration of such low levels with sufficient accuracy.

Taking into account the level of inoculation (target between 50 – 200 cfu/g), to avoid being faced to low numbers (less than 10 colonies on a Petri dish, according to EN ISO 7218) when colony counting, it is possible:

- either to decrease the dilution factor of the initial suspension (e.g. $1/5^{th}$ instead of $1/10^{th}$),
- or to increase the volume of the suspension plated on the dishes (e.g. 2 ml on 2 plates of ∅ 140 mm or 2ml on 6 plates on ∅ 90 mm).

The background microflora that may be taken into account include mesophilic aerobic counts (EN ISO 4833) or a specific microflora of the food (e.g. lactic acid bacteria, *Pseudomonas* spp, yeasts, moulds). Methods used to enumerate these specific microflora should follow relevant EN ISO or national standards for the organism and food type concerned.

6.2.2.9 Calculation of the growth potential

For each batch, the growth potential Δ is calculated according to the formula:

$$\Delta = \log_{max} - \log_{i}$$

where $\log_{max}$ is the highest value of the *Lm* enumeration obtained from, at least, the 4 sampling points (excluding the sampling at t0), when one test unit is analysed per sampling point.

When more than one test unit is analysed per sampling point, then $\log_{max}$ is the highest mean value obtained from each of these 4 sampling points.

$\log_{i}$ is the mean value of the 3 test units analysed at time zero (t0)

The growth potential retained amongst all tested batches is the highest obtained Δ value.

In this first example, presenting the most cases, 3 batches are analysed (Table 5). For each batch, 5 sampling points are spread over the duration of the test and at each sampling point 1 test unit is analysed.

Table 5. Calculation of a growth potential from 3 batches- 5 sampling points -1 test unit per sampling point.

	Time 0 (t0)		Time 1	Time 2	Time 3	Time 4	Δ_{batch}	**Δ_{Food}**
	[Lm] $\log_{10}$ cfu/g	Mean ± sd*	[Lm] $\log_{10}$ cfu/g	[Lm] $\log_{10}$ cfu/g	[Lm] $\log_{10}$ cfu/g	[Lm] $\log_{10}$ cfu/g		
batch 1	2.15 2.18 2.08	2.14 ± 0.05	2.15	2.11	2.04	2.18	2.18 -2.14 = 0.04	**0.08**
batch 2	2.11 2.15 2.04	2.10 ± 0.06	2.18	2.10	2.11	2.08	2.18-2.10 = 0.08	
batch 3	2.16 2.26 2.18	2.20 ± 0.05	2.20	2.14	2.24	2.06	2.24 -2.20 = 0.04	

*sd : standard deviation

In this first example, the standard deviation (sd) between the 3 results at time 0 (t0) for batch 1, batch 2 and batch 3 are respectively 0.05, 0.06 and 0.05 $\log_{10}$. The inoculation step has been correctly performed (<0.3 $\log_{10}$), thus the results of the challenge test obtained for assessing the growth potential can be used.

In this example, the highest "Δ" value among the 3 batches is 0.08 $\log_{10}$.

The growth potential is below the criterion 0.5 $\log_{10}$ that determines if the RTE food is able or not to support growth of *Lm*. So, this RTE food does not support the growth of *Lm* and can be classified in category 1.3 of Regulation (EC) 2073/2005.

Note: Where 3 batches are analysed at the same time, 1 sampling point at t0 to calculate the standard deviation is sufficient.

This second example presents the results of a challenge test obtained from 3 batches, 5 sampling points and 3 test units per sampling point (because of the variability among the physico-chemical parameters in the studied product) (Table 6).

Table 6. Calculation of a growth potential from 3 batches- 5 sampling points -3 test units per sampling point.

	Time 0 (t0)		Time 1		Time 2		Time 3		Timet4		Δ batch	**Δ**
	[Lm]	Mean ± sd*	[Lm]	Mean	[Lm]	Mean	[Lm]	Mean	[Lm]	Mean		
batch 1	2.10 2.28 1.80	2.06 ± 0.24	2.90 1.98 3.10	2.66	3.00 3.86 3.95	3.60	4.12 4.24 4.18	4.18	4.98 4.75 4.73	4.82	4.82 -2.06 = 2.68	**3.30**
batch 2	1.48 2.36 2.18	2.00 ± **0.46**										
batch 3	2.16 2.26 2.18	2.20 ± 0.05	1.80 3.00 3.10	2.63	3.90 3.95 3.97	3.94	4.20 4.31 4.50	4.34	5.80 5.30 5.40	5.50	5.50 -2.20 = 3.30	
batch 4	2.20 2.04 1.96	2.06 ± 0.12	2.28 2.72 2.85	2.62	3.14 3.53 3.26	3.31	3.25 4.0 3.11	3.45	4.42 4.18 4.50	4.36	4.36-2.06 = 2.30	

*sd: standard deviation

At time 0, the standard deviation of batch 2 is equal to 0.46 log10. It is higher than the tolerance of 0.3 log10, meaning that the inoculation step was not carried out correctly. Thus the challenge test on batch 2 has been stopped, because starting a challenge test with such a difference between the inoculated samples is not acceptable. A challenge test on another batch (named batch 4 in the table) was relaunched.

Considering the results of the challenge test carried out on batch 1, batch 3 and batch 4 of Table 6, the growth potential of *Lm* in the tested product can be assessed.

The highest "Δ" value among the 3 batches is 3.30 log10. The growth potential is above the criterion 0.5 log10 that determines if the RTE food is able or not to support growth of *Lm*. So, this RTE food supports the growth of *Lm* and is classified in category 1.2 of Regulation (EC) 2073/2005.

6.2.2.10 Application of results

The FBO is responsible for the use of the results of the challenge test. The results from a challenge test assessing the growth potential will inform the FBO on the two following points:

- The ability of the product to support growth of *Lm*

The first question to sort out is whether the food is able or not to support the growth of *Lm* if:

- Δ is lower or equal to the limit of 0.5 $\log_{10}$, then it is assumed that the food is not able to support the growth of *Lm* (Category 1.3 of Regulation (EC) No 2073/2005);

- Δ is higher than the limit of 0.5 $\log_{10}$, then it is assumed that the food is able to support the growth of *Lm* (Category 1.2 of Regulation (EC) No 2073/2005).

- The estimation of the growth of *Lm* based on the obtained growth potential value

In the cases in which it is assumed that the food is able to support the growth of *Lm*, the Δ value may be used to estimate the growth (see examples below):

highest concentration of *Lm* during the food shelf-life = initial concentration of *Lm* + Δ

In practice, the highest concentration of *Lm* obtained from the above calculation may be used to determine if the limit of 100 cfu/g is exceeded or not, along the entire shelf-life of the food.

- Examples

QUESTION 1: Does the food support the growth of *Lm*, according to the Δ value?

In example 1, the growth potential is:

Δ = 0.08 $\log_{10}$

Δ is below 0.5 $\log_{10}$, then it is assumed that the food does not support growth of *Lm*. It can be classified in category 1.3 of Regulation (EC) 2073/2005

In example 2, the growth potential is:

Δ = 3.30 $\log_{10}$

Δ is much higher than 0.5 $\log_{10}$, then it is assumed that the food supports growth of *Lm* and belongs to category 1.2 of Regulation (EC) 2073/2005.

Thus, to ensure that *Lm* contamination will not exceed the limit of 2 log cfu/g at the end of the shelf-life, the initial concentration of *Lm* (Ci) should be very low, that means below 0.05 cfu/g (Ci = 2.0 - 3.30 = -1.3 log cfu/g)

QUESTION 2: What is the highest concentration of *Lm* along the entire shelf-life of the food, based on the growth potential obtained?

Example 1: The growth potential is:

$\Delta = 0.7 \log_{10}$

and the initial concentration of *Lm* in the product is Ci = 1 $\log_{10}$ cfu/g (10 cfu/g)

Δ is higher than 0.5 $\log_{10}$, then it is assumed that the food supports growth of *Lm.* This Δ-value can be used for further calculations. The highest concentration of *Lm* during the shelf-life of the food can be estimated using the following equation:

highest concentration of *Lm* = initial concentration of *Lm* + Δ

highest concentration of *Lm* = 1 $\log_{10}$ cfu/g + 0.70 $\log_{10}$ = 1.70 $\log_{10}$ cfu/g

(below the regulatory limit of 2 $\log_{10}$ cfu/g)

6.2.2.11 Test report

Refer to EN ISO 20976-1 for all the points to include in the test report.

It is important to mention in the test report that the results of the challenge tests on the growth potential apply only to the tested product, under the storage conditions (time/temperature) applied. Any change to the product recipe, the production process, the storage conditions (time /temperature) would invalidate the results of the shelf-life study and would require it to be conducted again.

If the challenge test is performed for a single product or representing a group of products, this shall be indicated in the test report.

The exploitation of the results (when given by the competent laboratory) are out of the scope of this test report. It should be part of the shelf-life study report prepared by the FBO.

6.3 Challenge test assessing maximum growth rate

6.3.1 Introduction

A microbiological challenge test assessing maximum growth rate is a laboratory-based study which estimates the growth of *Lm* in a food artificially contaminated with one *Lm* strain at a time and stored at a constant temperature (usually between 6° and 10°C) throughout the duration of the test.

The maximum growth rate μ_{max} is the kinetic parameter that characterises the increase of the *Lm* population in the exponential phase of the growth curve on the natural logarithm (Ln) scale.

From the experimental growth curve obtained by plotting the concentration of *Lm* (expressed in $\log_{10}$ cfu/g) versus the time (expressed in hours or days) the maximum growth rate is noted $V_{max.}$

To switch from one to the other, the relation is $\mu_{max} = V_{max} * Ln (10) = V_{max} * 2.3$.

In scientific literature, and in most of the predictive microbiology tools, the maximum growth rate is usually reported as μ_{max}, to avoid confusion it is essential to pay attention to the units.

The duration of a challenge test assessing the maximum growth rate may be different to the duration of the shelf-life of the product. Its duration is defined by the time required to construct the growth curve at the selected temperature.

The advantage of the challenge test assessing maximum growth rate is the flexibility: when determined in a given condition of time and temperature, the growth rate can be estimated in other time/temperature conditions without the needs to conduct another challenge test, given that the cardinal values of the studied strain are known.

The drawback of this challenge test is the lack of consideration to the effect of the lag phase, which can lead to a different estimated concentration of *Lm* depending on whether it is taken into account or not. The most extreme example is probably HPP treated food, where there will be a long lag phase, but an exponential growth as large as if the food was not heat treated.

6.3.2 Protocol of a challenge test to assess maximum growth rate

Note: For the sections on "Inoculation of the test units" and “Measurement of physico-chemical parameters of the product”, please refer to the part on the growth potential because they are identical. The sections below are specific to the protocol to assess the maximum growth rate.

6.3.2.1 Number of batches

As a rule, at least 3 batches have to be tested for determining the microbiological shelf-life of a RTE food, in order to be able to capture the inter batch variability.

However, based on the calculator tool "Inter-Batch Physico-Chemical Variability calculator" available at http://standards.iso.org/iso/20976/-1/ed-1/en, provided with EN- ISO Standard 20976-1, challenge test can be run using only one batch, if:

- the FBO can provide data on the factors characterising a product within a batch (intra-batch variability) and between batches(inter-batch-variability);
- the physico-chemical parameters, pH and a_w , are the main factors that impact the growth of *Lm* in the product;
- the impact of inter-batch variability of these physico-chemical parameters on the growth of *Lm* is deemed to be not significant (result of the calculator tool).

The use of this calculator tool is only suitable for challenge tests conducted for assessing the maximum growth rate.

6.3.2.2 Choice of strains

A single strain is used per challenge test, so it is important to select the use of a strain with known growth characteristics, and suitable for the type of product studied.

This strain can be selected from the EURL *Lm* set of strains characterised by their maximum growth rate at low temperature, pH and a_w. It can be ordered from the *Lm* National Reference Laboratories.

The use of strains characterised by their cardinal values (according to standard ISO/NP 23961 on the "Determination and use of cardinal values" still under development) is advised. Using such strains, will allow predicting the growth of the strain in the studied food under different environmental conditions, not tested during the challenge test, more accurately.

6.3.2.3 Preparation of the inoculum

Conditions for preparation of the inoculum are identical to those described for the challenge test assessing the growth potential, except that the strain is cultured and used individually.

6.3.2.4 Inoculation of the test units

Conditions for inoculation of the test units are identical to those described for the challenge test assessing the growth potential, except that the inoculation is performed with one strain and not with a mixture of strains for each growth curve.

6.3.2.5 Number of units - Number of sampling points

Refer to EN ISO 20976-1 for the parts related to:

- **The number of units to be prepared** (number of test units to be inoculated, number of control units and food control samples);
- **The number of sampling points.**

See also annex 10.14 of the present document.

6.3.2.6 Storage conditions

The challenge test is conducted at one constant temperature. The storage temperature should be between 6° and 10°C. In case of contaminated products with lactic acid bacteria then the temperature should not be above 8°C, to avoid growth inhibition by lactic acid bacteria at higher temperatures.

The time of the experiment should be long enough to build the growth curve and this time can be longer or shorter than the studied shelf-life.

6.3.2.7 Microbiological analyses

Refer to section 6.2.2.8 of the part on the growth potential, except for the detection of *Lm* performed in the food control sample at time 0. When *Lm* is detected on the studied batch in the food control sample, then the challenge test will be stopped (because of the single strain inoculation in a challenge test assessing the maximum growth rate).

6.3.2.8 Calculation of the maximum growth rate

For each growth curve (one growth curve per batch), the maximum growth rate can be easily estimated by fitting a primary model (non-linear regression) on all the experimental points of the growth curve. This fitting can be done by using free available predictive microbiological software, for example: DMFit from ComBase software (www.combase.cc) or Curve fitting from Sym'Previus (www.symprevius.eu).

The estimated maximum growth rate of the tested product determined from the challenge test is equal to the average (expressed with its standard deviation) of the maximum growth rate values obtained from the growth curves (at least one per batch).

To construct the growth curve, it is necessary to pay attention to the distribution of the experimental points over time, in order to be able to accurately estimate the maximum growth rate. For a good fitting of the primary model to the experimental data points, it is important to have one point at the beginning of the stationary phase and one later in the same phase (Figure 3).

Depending on the software used to estimate the maximum growth rate, the result is not given in the same unit. On DMFit, if the input data is in $\log_{10}$ cfu/g, then V_{max} is calculated in $\log_{10}$ cfu/g, it is then

necessary to multiply it by 2.3 to obtain the correspondent μ_{max}. On Sym'Previus, the input data is in $\log_{10}$ cfu/g and the maximum growth rate (μ_{max}) is given and always expressed in Ln cfu/g.

Note that, if the increase in the microbial population observed from the growth curve is small (< 1 log cfu/g), this does not allow a reliable determination of μ_{max}. In that case, it could be recommended to build another growth curve at a higher temperature for the fitting or to run a growth potential study.

If a product is suspected to be unable to support the growth of *Lm* based on the physico-chemical characteristics, then it is not possible to reliably estimate a maximum growth rate. A challenge assessing the growth potential should be performed to prove this.

Finally, it is important to evaluate the uncertainty around the estimated maximum growth rate (μ_{max}) reflected by the standard error (se) or the confidence interval (CI) around the μ_{max} estimate. It is important that the standard error does not exceed 20% of the μ_{max} estimate. If not, this means that there is high uncertainty associated with μ_{max}, and this result should be interpreted with caution.

6.3.2.9 Application of results

The FBO is responsible for the use of the results of the challenge test.

The maximum growth rate can be used to assess the increase in *Lm* population during the shelf-life of the product under different storage temperatures.

From the challenge test growth kinetic obtained at one constant temperature ($T°_{CT}$), it is possible to estimate a growth rate at another temperature (T°).

The growth rate determined by fitting a primary model (e.g. DMFit) to the growth kinetic is noted growth $rate_{CT}$. Then, the calculation of a growth rate in the same food (same physico-chemical characteristics) at another temperature T° can be obtained using secondary models.

The following simplified equation (Ratkowski et al., 1982) (Mejlholm et al., 2010) of the square root secondary model (one of all available secondary models) can be used (if only the temperature is considered in the simulation and if T° and T_{CT} are both below 25°C). This formula does not take the effect of lactic acid bacteria on the growth inhibition of *Lm* into account. Therefore the formula is not be suitable for products with high level of lactic acid bacteria. A more complete model should be used in this case.

$$\text{growth rate}_{T°} = \text{growth rate}_{CT} \cdot \frac{(T° - T_{min})^2}{(T_{CT} - T_{min})^2}$$

where T_{min} is the minimal growth temperature for *Lm* (-2°C is the default value given in Table 1 that can be used for all strains as a generic value).

Increase in *Lm* (in log_{10}) for a storage time d_1 (in days) at T_{CT} = growth $rate_{CT}$ (log_{10} cfu/g per day) x d_1

Increase in *Lm* (in log_{10}) for a storage time d_2 (in days) at T° = growth rate $_{T°}$ (log_{10} cfu/g per day) x d_2

Noted: For this calculation the maximum growth rate must be expressed in log_{10} (= V_{max}).

The growth simulation can 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.

The cardinal model is another secondary model that can be used. In that case, to better predict the growth of the specific *Lm* strain inoculated in the product, it is advised to determine the cardinal values (T_{min}, T_{opt} and T_{max}) of this strain based on the standard ISO /NP 23691 "Microbiology of the food chain-determination and use of cardinal values" and introduce these values in the cardinal model. User friendly tools can be used for this purpose (e.g. Growth Simulation from Sym' Previus software (www.symprevius.eu)).

Example 1. Estimation of the increase of *Lm* population without lag time

- **Data**:

- Product with a shelf-life of 9 days ("day 0" is the day of production);
- Storage conditions: 4°C for 3 days (d1) and 8°C for 6 days (d2);
- The challenge test was performed on this product at T_{CT} = 8°C.

The maximum growth rate (with its standard error) estimated at 8°C from the fitting is:
Growth $rate_{CT}$ = 0.764 log_{10} cfu/g . d^{-1} ± 0.0367 (Figure 3).

The visual look of the curve gives confidence that the datapoints describe the whole sigmoid curve. If we divide the standard error linked to the growth rate by its value we obtain: 0.0367/0.764 = 4.8%. This is below the 20% uncertainty threshold and gives confidence that this growth rate estimate is accurate.

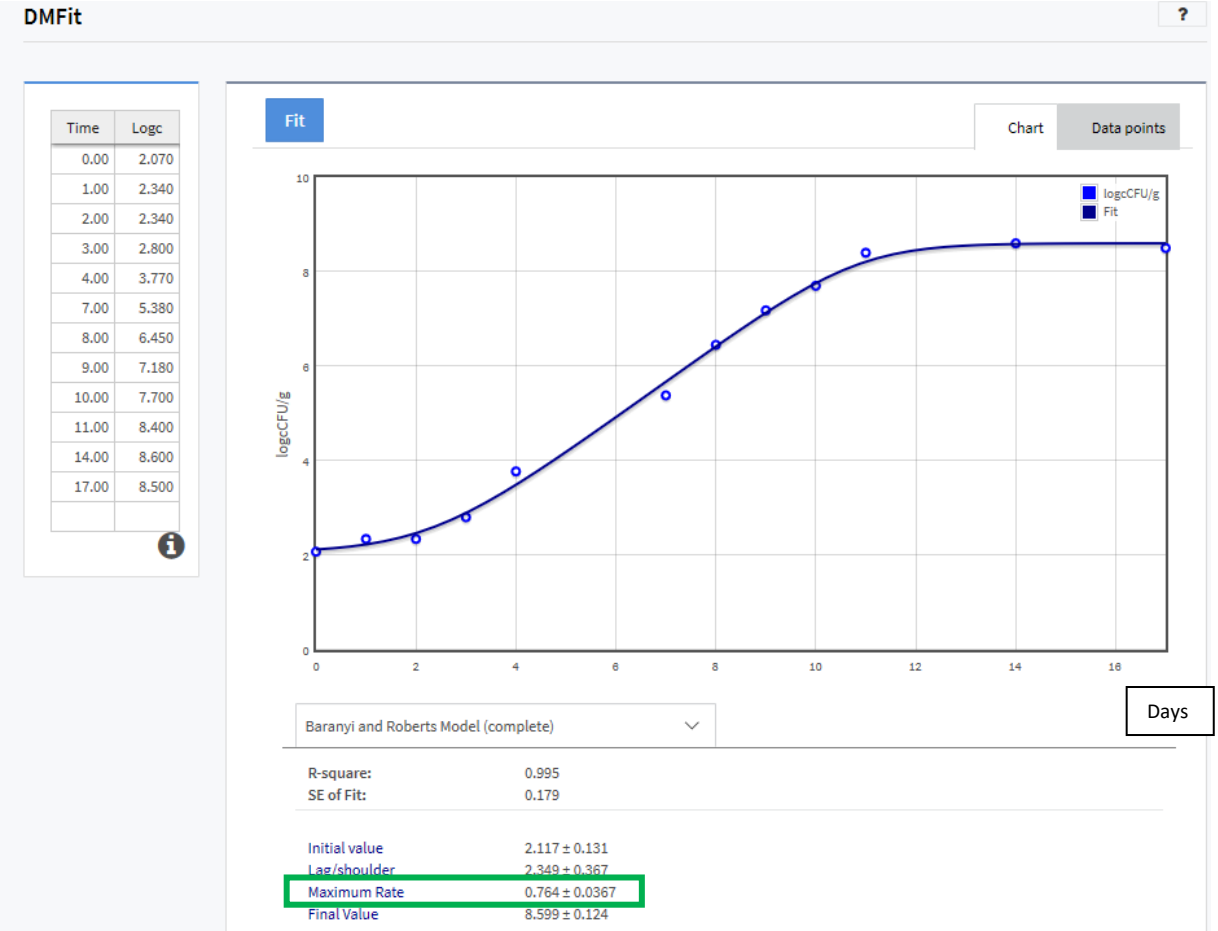

Figure 3. Fitting with DMFit from ComBase software (www.combase.cc)

The use of the simplified equation of the square root secondary model enables to estimate the growth rate at T° = 4°C

$$\text{growth rate } (T°) = \text{growth rate}_{CT} \cdot \frac{(T° - T_{min})^2}{(T_{CT} - T_{min})^2}$$

The maximum growth rate at 4°C estimated from the above equation of the secondary model is:
Growth rate T° (4°C) = 0.764 x $(4 - (-2))^{\wedge 2}/(8-(-2))^{\wedge 2}$ = 0.275 $\log_{10}$ cfu/g . d^{-1}

- **Question 1:** what is the estimated growth of *Lm* in the product during the shelf-life?

Estimated growth of *Lm* during the shelf-life =

[(growth rate$_1$ (4°C) in $\log_{10}$ cfu/g per day) x d_1] + [(growth rate$_2$ (8°C) in $\log_{10}$ cfu/g per day) x d_2] where:

Estimated growth increase of *Lm* during the shelf-life = (0.275 x 3) + (0.764 x 6) = 5.42 $\log_{10}$

This simple calculation does not include a lag phase, nor a stationary phase (i.e. assumes an exponential growth throughout the length of the shelf-life) and consequently represent a worst-case scenario. To close this gap, predictive microbiological software can be used.

When including a lag time into the estimation of the shelf-life care should be taken, because of the difficulty to evaluate the physiological state of *Lm* when the contamination of the product occurs. The choice of including a lag phase should be justified/explained.

Example 2. Estimation of the increase in *Lm* population, where a lag time is included.

- **Data**:

- Product with a shelf-life of 9 days ("day 0" is the day of production);
- Storage at 4°C for 9 days;
- The challenge test was performed on this product at TCT = 8°C.

Based on the fitting obtained at 8°C, the lag time was estimated at 2.35 days.

The relationship, $\boxed{\text{lag x Growth rate} = h_0}$ (Baranyi and Roberts, 1994), is used to calculate the constant parameter h_0, from which the lag time at 4°C will be estimated.

According to the fitting at 8°C, $h_0 = 0.76 \times 2.35 = 1.79$.

Thus, the lag at 4°C = 1.79/0.275 = 6.5 days. This means that growth will only start after 6.5 days at 4°C, meaning that there will be no growth during the first 6.5 days at 4°C.

Then it is possible to evaluate the growth on the rest of the storage period of (9 - 6.5) = 2.5 days at 4°C.

The estimated growth increase of *Lm* during the shelf-life = (0) + (0.275 x 2.5) = 0.69 $\log_{10}$

6.3.2.10 Test report

Refer to EN ISO 20976-1 for all the points to be included in the test report.

The results of the challenge test apply to the product tested (μ_{max} being specific to the strain used and to the intrinsic and extrinsic specific characteristics of the studied product). However, it also provides useful data to simulate the effects of variation in these characteristics (pH, a_w, preservatives, temperature) of the studied products.

The exploitation of the results (when given by the laboratory, competent in predictive microbiology) are out of the scope of this test report. It should be part of the shelf-life study report prepared by the FBO.

7 Durability study

7.1 Introduction

A durability study related to *Lm* is a laboratory study conducted to determine the concentration of *Lm* at the end of the shelf-life, in a naturally contaminated product stored under reasonably foreseeable conditions from production to consumption.

The aim of durability studies is to estimate the proportion of RTE foods exceeding the quantitative limit of 100 cfu/g at the end of the shelf-life after a storage period reflecting the foreseeable conditions of distribution, storage and use.

This type of study is not suitable alone to validate the microbiological shelf-life of RTE foods related to L*m*, because of a low prevalence, low level of contamination and an heterogeneous distribution of *Lm* contamination in solid products. In most cases, results will be obtained from samples not contaminated with *Lm*, making it impossible to conclude on the evolution of *Lm* in the product. Combining durability studies with other studies, such as challenge tests or predictive microbiology, contribute to validate the shelf-life of a RTE food related to *Lm.*

In the context of *Lm*, a durability study can be suitable in the two following cases:

a/ for RTE food able to support *Lm* growth and frequently contaminated with low level of *Lm*, for the verification of the shelf-life.

b/ for RTE food where a batch is unexpectedly (accidentally) contaminated with *Lm*, in order to evaluate *Lm* growth when naturally contaminated.

7.2 Protocol for a durability study

When conducting a durability study, the following steps have to be considered:

- Description of the RTE food to be tested;
- Food sampling ;
- Storage of samples ;
- Microbiological analyses ;
- Results.

7.2.1 Description of the RTE food to be tested

See the paragraph 6.1 “Prerequisites before initiating a challenge test” describing the relevant information required by the laboratory.

7.2.2 Food sampling

Two food sampling procedure may be performed: A single random sampling or a directed sampling.

- Single random sampling (annex 10.15):
  This sampling method applies for RTE food able to support *Lm* growth and frequently contaminated with low level of *Lm*. This sampling should be repeated for different batches over time (same product, same production process) in order to obtain data that can be gathered to give more confidence in the obtained result.

- Targeted sampling:
  This sampling method applies to RTE food where a batch is detected unexpectedly contaminated (accidentally contaminated) with *Lm*, before being placed on the market. In that case it is recommended to take as many samples as possible from the contaminated batch, and as close as possible to the production date.

7.2.3 Storage of samples

See the part “Storage conditions” in paragragh 6.2.2.6 of “Challenge test assessing growth potential”.

7.2.4 Microbiological analyses

It is recommended to decrease the limit of the enumeration method to 10 cfu/g or even more lower, in order to have the best chance of getting a numerical result (x cfu/g) and not a truncated one at < 100 cfu/g.

a/ For RTE food able to support *Lm* growth and frequently contaminated with low level of *Lm*

Quantitative analyses are performed at the end of the shelf-life on all the units stored under reasonably foreseeable conditions, in order to assess whether the level of 100 *Lm*/g or ml is exceeded or not at the end of the shelf-life.

b/ For RTE food where a batch is unexpectedly (artificially) contaminated with *Lm*

At least, analyses are performed at the beginning (as close as possible to the production date) and at the end of the shelf-life. When possible, analyses at other intermediate dates providing information about *Lm* behaviour are recommended.

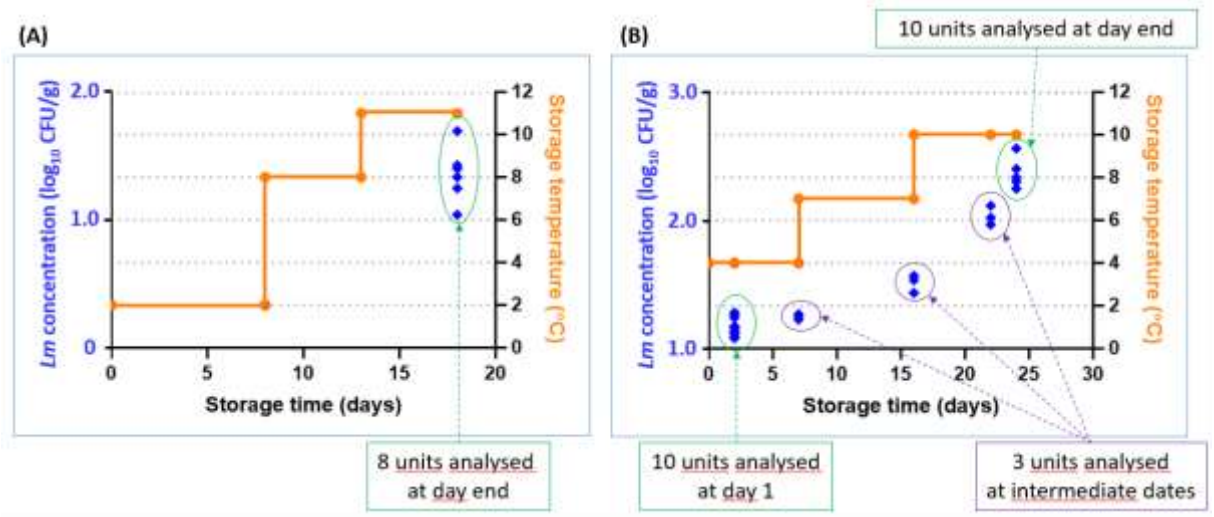

Figure 4. Examples of durability studies in the case of a batch frequently contaminated with low level of *Lm* (A) or a batch unexpectedly contaminated with *Lm* (B)

In Figure 4-A, all units are analysed at day end in order to get a more precise information on proportion of units that could be above 100 cfu/g at the end of the shelf-life. In Figure 4- B, ten samples were analysed at day 2, as soon as the batch has been detected unexpectedly contaminated by *Lm* with a level of contamination around 1 $\log_{10}$ cfu/g (10 cfu/g). Then at each storage temperature changes (reflecting the foreseeable storage conditions of the product) 3 samples were analysed. Ten samples were also enumerated for *Lm* at day 24 (end of the shelf-life).The increase in the *Lm* population in this naturally contaminated product is around 1.3 $\log_{10}$ cfu/g.

7.2.5 Results

For RTE food able to support *Lm* growth and frequently contaminated with low level of *Lm*, this interpretation can be facilitated by assessing the estimated proportion of units exceeding 100 cfu/g at the end of shelf-life, after a storage period reflecting the foreseeable conditions of distribution and storage, as described below.

From the number (n) of units taken randomly from a batch (of size N), the observed proportion (p) of units exceeding 100 cfu/g at the end of shelf-life is:

$$p = r / n \text{ (where } r \text{ is the number of test units above 100 cfu/g).}$$

To estimate, with a confidence interval (CI) at 95%, the proportion of units above 100 cfu/g in the whole batch population, the following calculator can be used:
http://www.causascientia.org/math_stat/ProportionCI.html.

This calculator provides two methods of calculation, the central confidence interval or the shortest confidence interval. Confidence intervals (CI) given by each method may be slightly different but are in the same order of magnitude. It is recommended to use the general one, the central confidence interval.

Table 7 points out the real importance of drawing from one batch a sufficient number of units, and/or to gather results previously obtained, in order to get a better estimation of the proportion of units above 100 cfu/g.

For example, when 10 units are analysed and that among those units none of them have a concentration of *Lm* > 100 cfu/g, then the estimated proportion of units > 100 cfu/g in the whole population can reach 28% (upper CI value). This estimated proportion is 13% (upper CI value) when 50 units are analysed and 2 units are above 100 cfu/g. These examples highlight that reporting the upper confidence interval value is of importance.

Table 7. Example of the estimated proportions of units > 100 Lm/g in the whole batch with regard to the number of analysed units

n number of analysed units	*r* number of units > 100 cfu/g	*p* observed proportion of units > 100cfu/g	Estimated proportion (with a CI at 95%) of units > 100 cfu/g in the whole batch
5	0	0%	[0% - 46%]
10		0%	[0% – 28%]
20		0%	[0% – 16%]
30		0%	[0% – 11%]
50		0%	[0% – 7%]
100		0%	[0% – 4%]
5	1	20%	[4% – 64%]
10		10%	[2% – 41%]
20		5%	[1% – 24%]
30		3%	[0.7% – 16%]
50		2%	[0.4% – 10%]
100		1%	[0.2% – 5%]
5	2	40%	[12% – 78%]
10		20%	[6% – 52%]
20		10%	[3% – 30%]
30		7%	[2% – 21%]
50		4%	[1% – 13%]
100		2%	[0.6% – 7%]

The more units are analysed, the narrower is the confidence interval. 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 product, same process.

Note: In the case of batch testing (official control), one of the criteria defined by Regulation No. 2073/2005 for RTE foods of category 1.2 (able to support the growth of *Lm*), is "n=5, c=0, m=M=100 cfu/g" at the time of consumption. When the limit defined by the criterion is exceeded, the product is considered to be unsafe and cannot be put on the market. Revision and improvement of the production process, reformulation and / or shelf-life reduction are thus required. However, such batch conformity controls are not in the scope of the present document.

7.2.6 Study report

The study report shall include all the information related to the five steps of a durability study:

- RTE food tested (identification, composition, shelf-life, physico-chemical and microbiological characteristics, production process, packaging, ...);
- Food sampling (identification of the batch tested, date of sampling, method used, number of samples tested);
- Storage conditions (time/temperature profile, temperature recording along the duration of the test);
- Microbiological analyses (analytical methods, date(s) of analyses and number of units/date(s));
- Results obtained for the tested batch (number of analysed units, number of units exceeding 100 cfu/g, the observed and estimated proportion (with a CI at 95%) of units above 100 cfu/g in the tested batch).

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ISO 11290-2 Microbiology of the food chain - Horizontal method for the detection and enumeration of *Listeria monocytogenes* and of *Listeria spp.* — Part 2: Enumeration method

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9 Definitions

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

Control unit: unit of food identical to the test unit but not artificially contaminated (used as a blank)

Food control sample: control sample not subject to any preparation and use to verify the representativeness of the production

Hygrometry: measurement of the moisture in air and gases.

Percentile: the x^{th} percentile of a set of values divides these values so that x% of the values lie below and (100-x)% of the values lie above. Examples: Ninety percent of the values lie at or below the ninetieth percentile, ten percent above it. The median of the values corresponds to the 50^{th} percentile, that is fifty percent of the values below the median and fifty percent above the median.

pH: measure of the concentration of acidity or alkalinity in an aqueous solution. 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: period of time for which a product remains safe and meets its quality specifications under reasonably foreseeable conditions of storage, distribution and use.

Validation: Obtaining evidence that a control measure or combination of control measures, if properly implemented, is capable of controlling the hazard to the specified level

Verification: The application of methods, procedures, tests and other evaluations, in addition to monitoring, to determine whether a control measure is or has been operating as intended.

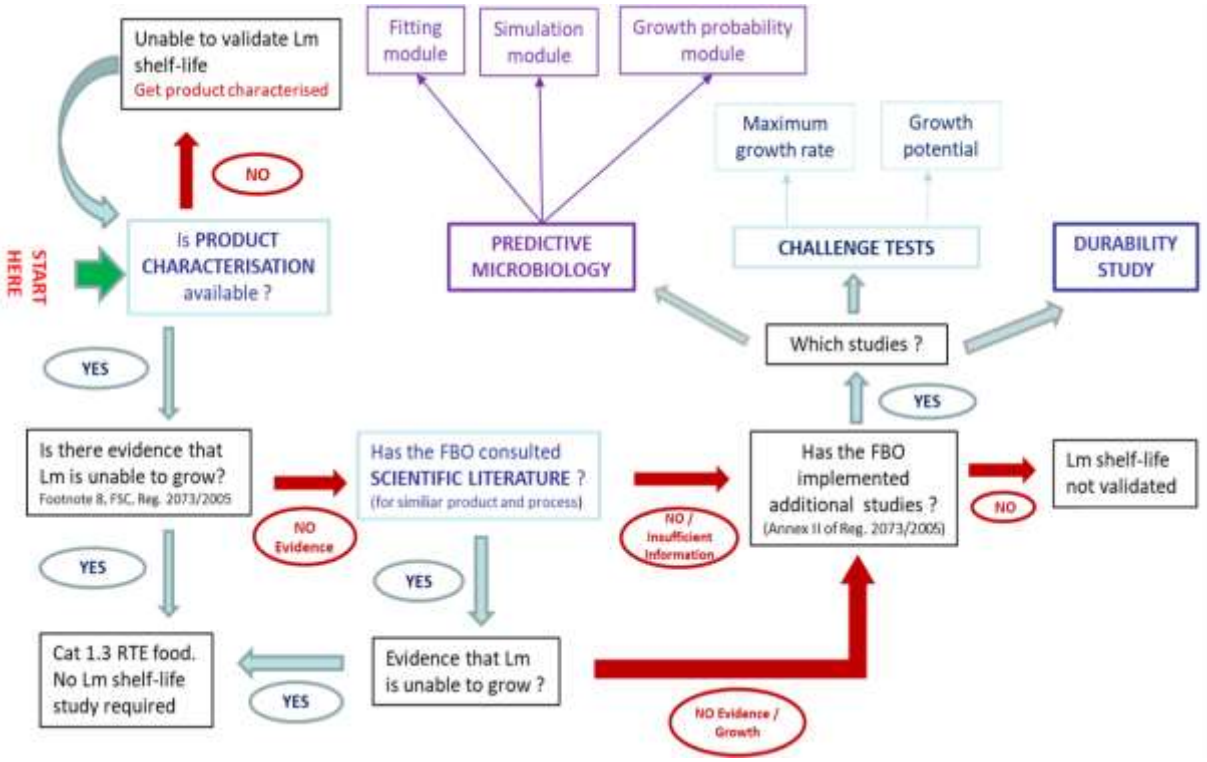
Water Phase Salt (WPS): Percentage of salt in the aqueous phase of a product

10 Annexes

10.1 Table outlining the benefits/limitations of challenge tests assessing growth potential, maximum growth rate and of durability studies.

Type of study	Benefits	Limitations
Challenge test - Growth potential (Δ)	Good tool to validate the shelf-life Calculation of Δ based on the use of a simple formula Value of Δ enables one to determine if RTE food supports or not *Lm* growth Experiments require less test units than experiments for the maximum growth rate	Results limited to conditions used in the study. Not possible to extrapolate. Need information on the time/temperature profile to simulate foreseeable storage conditions of RTE food Need incubators for reproducing with precision the defined temperature profile
Challenge test - Maximum growth rate (μ_{max})	Good tool to validate the shelf-life Possible to extrapolate the result to other conditions Experiment performed at a constant temperature chosen by the laboratory Gain in time for assessing a long shelf-life Use of μ_{max} in predictive microbiology models estimates *Lm* level of contamination in various environmental conditions	Need to have knowledge in predictive microbiology and in the use of predictive microbiological software Need to carry out more experiments at timed intervals hence more test units are required. Strains are tested individually When no lag-phase is taken into account, thus such test may overestimate *Lm* concentration in the tested product
Durability study	Good tool to verify the established shelf-life Easy to implement at the laboratory, no specific equipment needed No bias concerning the physiological state of the bacteria in food because of the natural contamination Possible to gather durability studies to increase the level of confidence in the established shelf-life	Not suitable alone to validate the microbiological shelf-life of RTE foods Impossible to test all the foreseeable storage conditions Impossible to extrapolate the results to other conditions Need information on the time/temperature profile to simulate foreseeable storage conditions of RTE food Need to get a significant number of results to have a greater confidence in statistical interpretation

10.2 Flow diagram to establish and verify the shelf-life of ready to eat food with respect to *Listeria monocytogenes*



10.3 List of parameters characterising the product that have an impact on the growth of *Lm*

Intrinsic factors

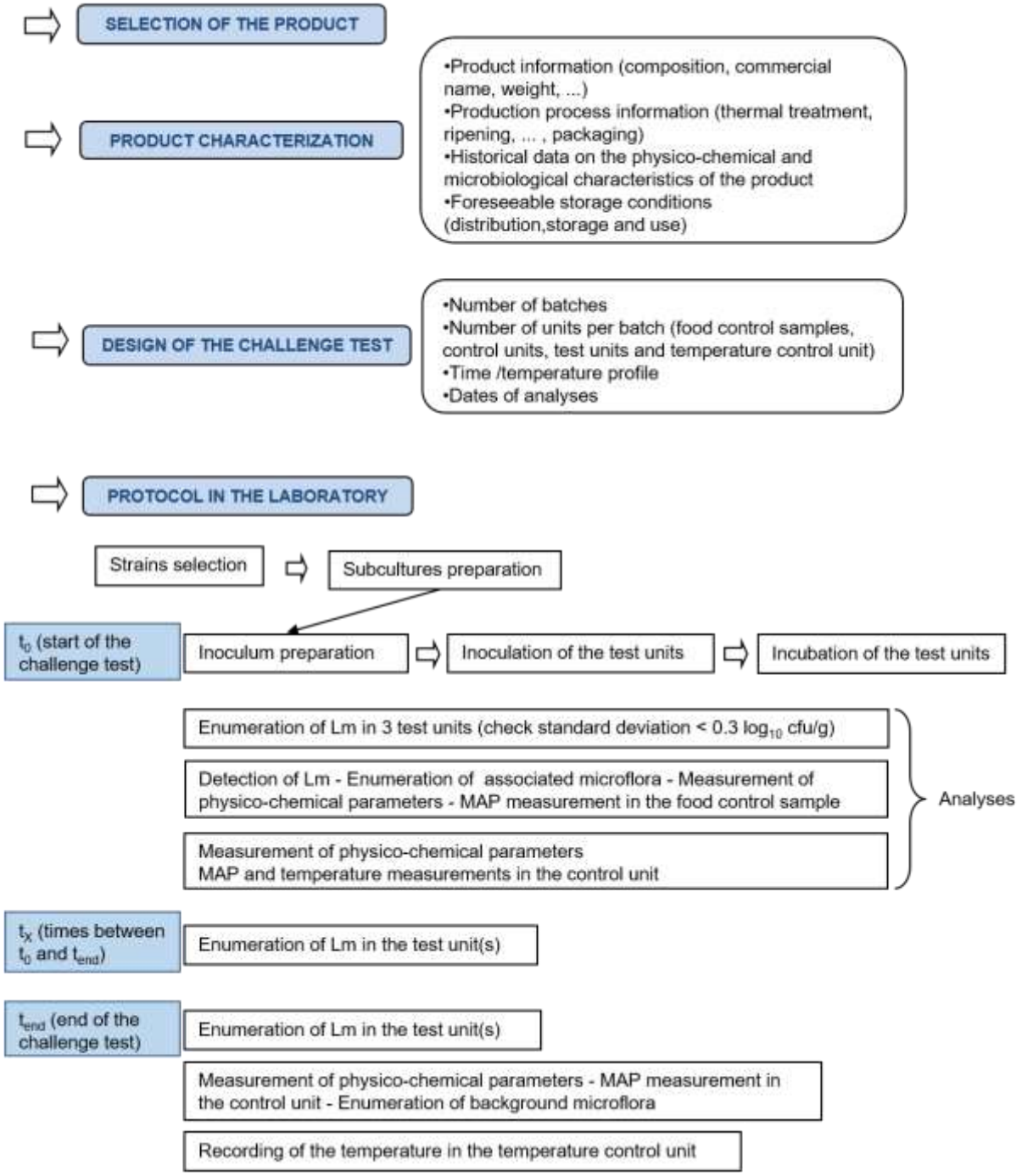
- pH
- a_w (water activity)* or salt/sugar and moisture contents
- Organic acids, Nitrite
- Preservative content
- Background microflora
- Structure of the food

*) a_w (water activity) of a food
- defined as the ratio: vapor pressure of water in a food (p)/vapor pressure of pure water (po) at the same temperature
- represents the amount of free water in the food, available to support the growth of bacteria

Extrinsic factors

- Temperature of cold chain
  - From manufacturer
  - To intermediate storage, transport, retail and consumer
- Relative air humidity
(throughout storage for unpacked products)
- Packaging conditions of the end product
(air packed, vacuum, MAP, gas permeability of the packaging material)
- Gas composition
(for products under MAP)

10.4 Flow diagram describing schematically the steps from FBO historical data to test in the laboratory



10.5 Set of *L. monocytogenes* strains with their growth characteristics

The strains set of EURL *Lm* was classified according to their growth rates related to origins, conditions of temperature, pH and a_w, and genoserotypes. More details are described in the report dedicated to strains set for challenge tests available at https://eurl-listeria.anses.fr/

Table 8. Choice of strains according to growth abilities related to origins, conditions and genoserotypes

Origin	Meat products		
Genoserotype	Low a_w (a_w = 0.95)	Low pH (pH = 5)	Low temperature (T = 8°C)
II	12MOB045LM 12MOB046LM	12MOB045LM 12MOB046LM	12MOB045LM 12MOB046LM
IV	12MOB085LM 12MOB089LM	12MOB112LM 12MOB089LM	12MOB085LM 12MOB089LM
Origin	Fish products		
Genoserotype	Low a_w(a_w = 0.95)	Low pH (pH = 5)	Low temperature (T = 8°C)
II	12MOB101LM 12MOB100LM	12MOB101LM 12MOB100LM	12MOB099LM 12MOB101LM
IV	12MOB103LM 12MOB102LM	12MOB103LM 12MOB102LM	12MOB102LM 12MOB107LM
Origin	Dairy products		
Genoserotype	Low a_w (a_w = 0.95)	Low pH (pH = 5)	Low temperature (T = 8°C)
II	12MOB098LM 12MOB118LM	12MOB118LM 12MOB098LM	12MOB098LM 12MOB079LM
IV	12MOB096LM 12MOB106LM	12MOB097LM 12MOB096LM	12MOB096LM 12MOB105LM
Origin	Other products		
Genoserotype	Low a_w (a_w = 0.95)	Low pH (pH = 5)	Low temperature (T = 8°C)
II	12MOB048LM 12MOB047LM	12MOB051LM 12MOB047LM	12MOB049LM 12MOB047LM/ 12MOB051LM
IV	12MOB050LM 12MOB052LM	12MOB050LM 12MOB052LM	12MOB052LM 12MOB050LM

How to use this table?

Example 1: if the product to be tested comes from dairy products, is rather acid (pH ≤ 5), then the chosen strain could be 12MOB118LM or 12MOB098LM or 12MOB097LM or 12MOB096LM.

Example 2: if the product to be tested comes from meat products, is neither acid (pH > 5), neither with a low a_w (a_w > 0.95), then the chosen strain could be 12MOB045LM or 12MOB046LM or 12MOB085LM or 12MOB089LM.

10.6 Example of preparation of the inoculum for the challenge test

A. *Preparation of subcultures for strain 1*

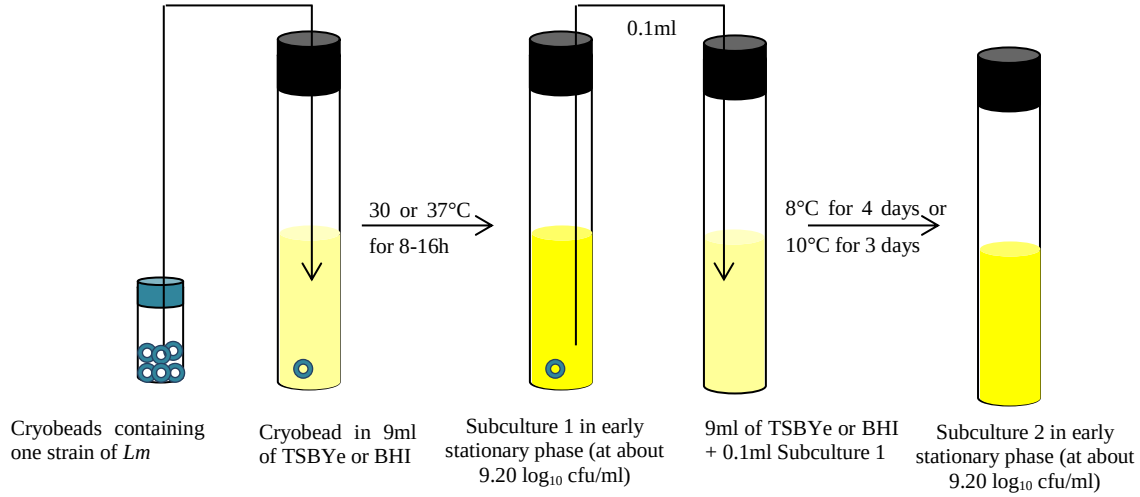


Figure 5: Preparation of the 2 subcultures for each strain

Process is repeated for strain 2 and other strains if used. Values given are for EURL *Lm* strains.

B. *Preparation of the inoculum for challenge test assessing growth potential*

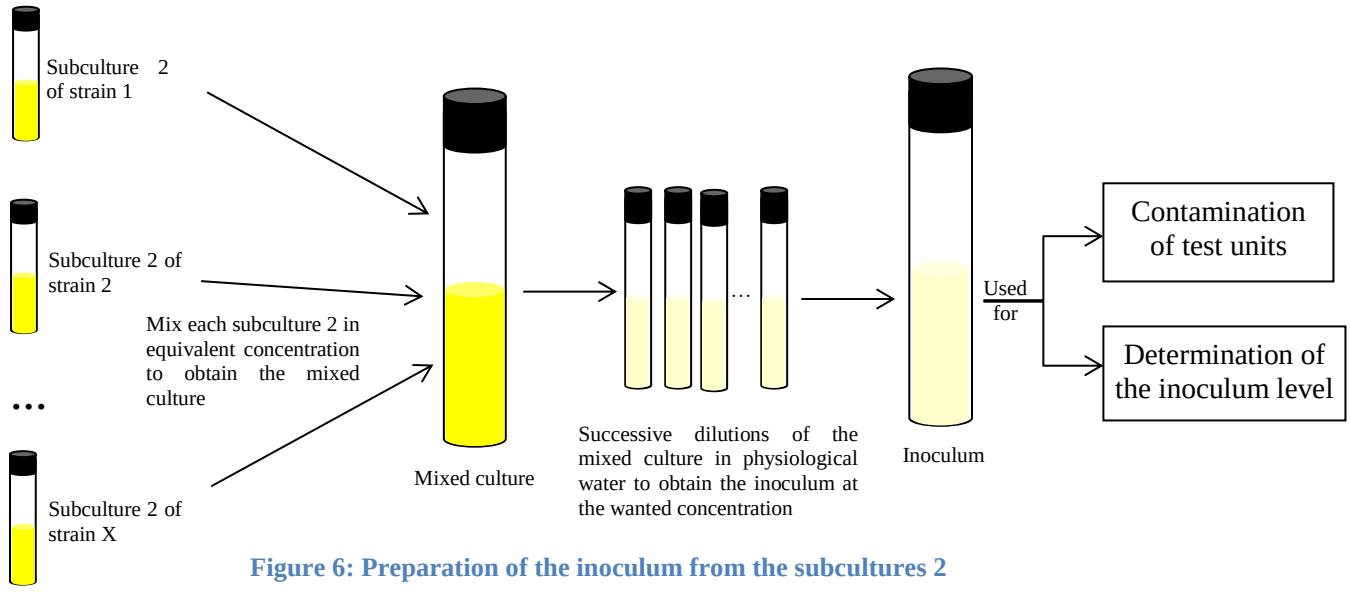


Figure 6: Preparation of the inoculum from the subcultures 2

C. *Preparation of the inoculum for challenge test assessing maximum growth rate*

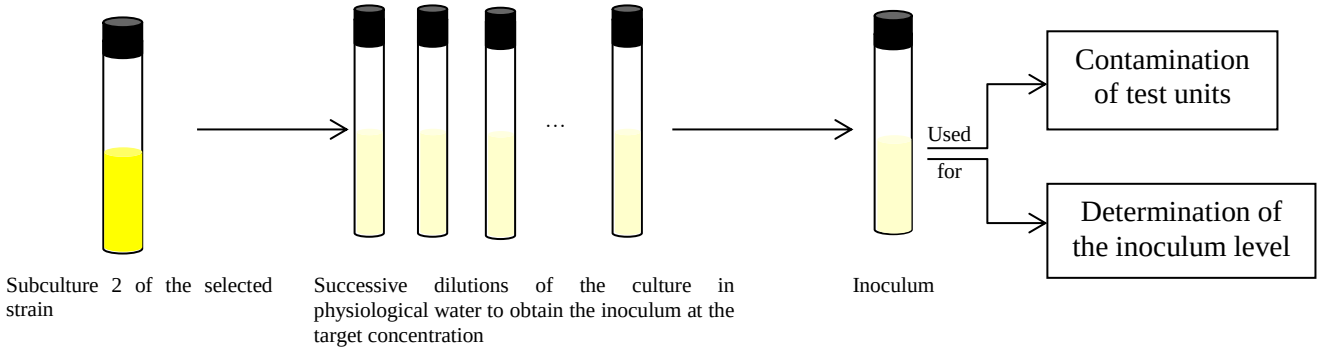


Figure 7: Preparation of the inoculum from the subculture 2 of a single strain

D. *Method to obtain the targeted concentration of the inoculum with a numerical example*

The mixed culture for challenge test assessing growth potential has an estimated concentration of 9.2 $\log_{10}$ cfu/ml, that is $1.58.10^9$ cfu/ml.

The targeted concentration in the whole matrix is 100 cfu/g.

The mass of the whole matrix is 650g. The volume of the inoculum to be introduced in the food matrix should not exceed 1% of the mass of the whole matrix; the maximum volume of the inoculum is 6.5ml.

It is necessary to dilute four times by decimal dilutions the mixed culture to come close to the required concentration of the inoculum in the whole matrix: $C_{\text{mixed culture diluted}} = 1.58.10^5$ cfu/ml.

It is necessary to prepare a suitable quantity of inoculum in order to be able to contaminate the whole matrix. For example 10 ml, so the concentration of the inoculum is $1.58.10^4$ cfu/ml.

The next step is to determine the required volume of the inoculum in order to contaminate the 650 g of the matrix. It is known that:

$$C_{\text{inoculum}} \times V_{\text{inoculum}} = C_{\text{whole matrix}} \times M_{\text{matrix}}$$

$$V_{\text{inoculum}} = (C_{\text{whole matrix}} \times M_{\text{matrix}}) / C_{\text{inoculum}}$$

$$V_{\text{inoculum}} = (100 \text{ cfu/g} \times 650 \text{ g}) / 1.58.10^4$$

$$V_{\text{inoculum}} = 4.1 \text{ ml}$$

In summary:

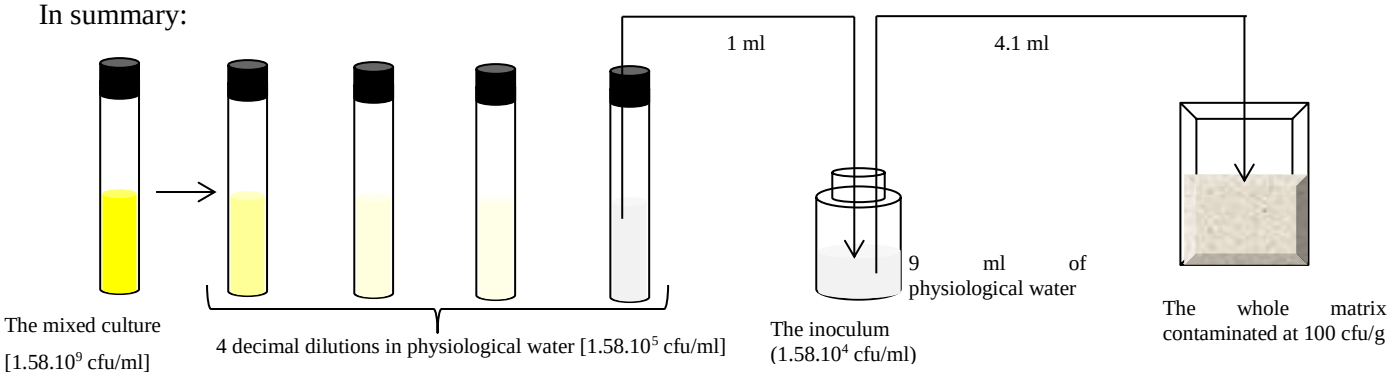


Figure 8: From the mixed culture to the inoculation of the whole matrix

The method to obtain the targeted concentration of the inoculum is the same for challenge test assessing the maximum growth rate, except that the starting point is not a mixed culture of strains but the second subculture of one strain.

10.7 Some examples of contamination techniques

Test units can be contaminated in depth or on surface.

This paragraph gives some examples of a couple of matrix and inoculation techniques:

- In depth: a semi-liquid product in small quantity (20 g) in a sterile bag

  for example 20 g of custard contaminated by a pipetted volume

- In depth: a semi-liquid product in large quantity (≈500 g) with a blender bowl and then divided in x samples of x g

  for example custard in large quantity contaminated by a pipetted volume

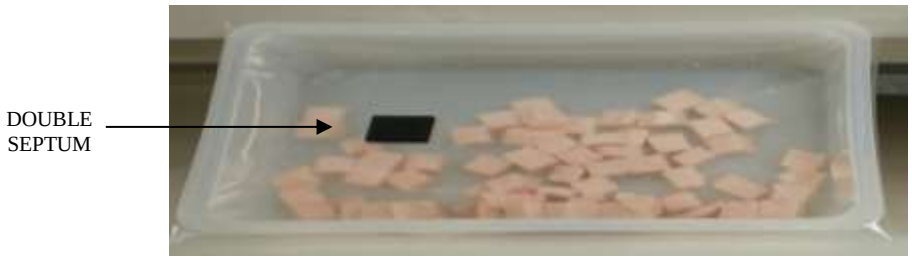
- At the surface: a sliced product

  for example a slice of smoked salmon contaminated with 5 spots of 20 µl on half of the disk's surface and then the disk is folded over. A spreader is used to improve the distribution of the inoculum.

- At the surface: a solid product of small pieces

  for example shredded ham contaminated at the surface of pieces with a graduated syringe through a septum. This septum is immediately recovered by a second septum in order not to break packaging atmosphere and maintaining the exact gas conditions.

  Note: It is possible to divide the inoculum into 2 parts and dispatched through 2 septums. The inoculum could be divided into more parts and dispatch through more septums. After inoculation, test units are shaken a lot in order to distribute homogeneously the inoculum.



10.8 Examples on the total number of units required for a challenge test assessing the growth potential

Table 9. Example 1: Products under air or vacuum packed – 3 batches analysed

Type of units	Type of analysis	Number of units and date of analyse per batch	
Test units	Enumeration of *L. monocytogenes*	**7**	3 test units at t_0 and 1 test unit at 3 intermediate dates and 1 at t_{end}
Food control samples	Detection of *L. monocytogenes*	**1**	1 at t_0
	Measurement of physico-chemical characteristics		
	Enumeration of the associated microflora		
Control units	Measurement of physico-chemical characteristics	**2**	1at t_0 and 1 at t_{end}
	Enumeration of the associated microflora		
	Temperature control	**1**	all along the test
Total number of units		**11 ***	

Total number required for 3 batches	**33 ***

(*) According on the amount of product in the units, this total number might need to be increased in order to have sufficient quantity of product to perform all the required analyses

Table 10. Example 2: Products under MAP – 3 batches analysed

Type of units	Type of analysis	Number of units and date of analyse per batch	
Test units	Enumeration of *L. monocytogenes*	**7**	3 test units at t_0 and 3 intermediates and 1 at t_{end}
Food control samples	Detection of *L. monocytogenes*	**2**	1 at t_0
	Measurement of physico-chemical characteristics		1 at t_0
	Enumeration of the associated microflora		1 at t_0
	MAP measurement		1at t_0 and 1 at t_{end}
Control units	Measurement of physico-chemical characteristics	**2**	1at t_0 and 1 at t_{end}
	Enumeration of the associated microflora		
	MAP measurement		
	Temperature control	**1**	all along the test
Total number required per batch		**12***	

Total number required for 3 batches	**36***

(*) According on the amount of product in the units, this total number might need to be increased in order to have sufficient quantity of product to perform all the required analyses

Table 11. Example 3: Products under air or vacuum packed – 3 batches analysed – Measurements of physico-chemical parameters outsourced

Type of units	Type of analysis	Number of units and date of analyse per batch	
Test units	Enumeration of *L. monocytogenes*	**7**	3 test units at t_0 and 3 intermediates and 1 at t_{end}
Food control samples	Detection of *L. monocytogenes*	**1**	1 at t_0
	Enumeration of the associated microflora		1 at t_0
	Measurement of physico-chemical characteristics	**2** (outsourced)	1at t_0 and 1 at t_{end}
Control units	Enumeration of the associated microflora	**2**	1at t_0 and 1 at t_{end}
	Measurement of physico-chemical characteristics	**2** (outsourced)	
	Temperature control	**1**	all along the test
Total number required per batch		**15***	

Total number required for 3 batches	**45***

(*) According on the amount of product in the units, this total number might need to be increased in order to have sufficient quantity of product to perform all the required analyses

Table 12. Example 4: Products under air or vacuum packed – 3 batches analysed - 3 test units analysed per sampling points

Type of units	Type of analysis	Number of units and date of analyse per batch	
Test units	Enumeration of *L. monocytogenes*	**15**	3 test units for each of the 5 sampling points
Food control samples	Detection of *L. monocytogenes*	**3**	1 at t_0
	Measurement of physico-chemical characteristics		3 at t_0
	Enumeration of the associated microflora		3 at t_0
Control units	Measurement of physico-chemical characteristics	**3**	3 at t_0 and t_{end}
	Enumeration of the associated microflora		
	Temperature control	**1**	all along the test
Total number required per batch		**22***	

Total number required for 3 batches	**66***

(*) According on the amount of product in the units, this total number might need to be increased in order to have sufficient quantity of product to perform all the required analyses

10.9 Example of the impact of storage temperature on the shelf-life

Temperature during the shelf-life is a critical part of the challenge test assessing the growth potential. This is illustrated below on a meat product stored at different temperatures:

- Scenario #1: at a constant temperature of 4°C;
- Scenario #2: includes 3 steps (one third of the shelf-life for each step), (i) 4°C to mimic storage/transportation from plant to retail, (ii) 7°C to mimic storage at retail and (iii) 10°C to mimic storage at consumer;
- Scenario #3: includes 3 steps (one third of the shelf-life for each step), (i) 7°C to mimic storage/transportation from plant to retail, (ii) 7°C to mimic storage at retail and (iii) 10°C to mimic storage at consumer.

Shelf-life of the product: 30 days.

Physico-chemical characteristics of the product:

- pH = 6.1 and
- aw = 0.978.

Packaging of the product: 50% CO_2 / 50% N_2.

Contaminated portion: 100g.

Mean initial contamination level of *Listeria monocytogenes* in this product: -2 $\log_{10}$ cfu/g.

The shelf-life of the product is estimated for each scenario (Figure 9).

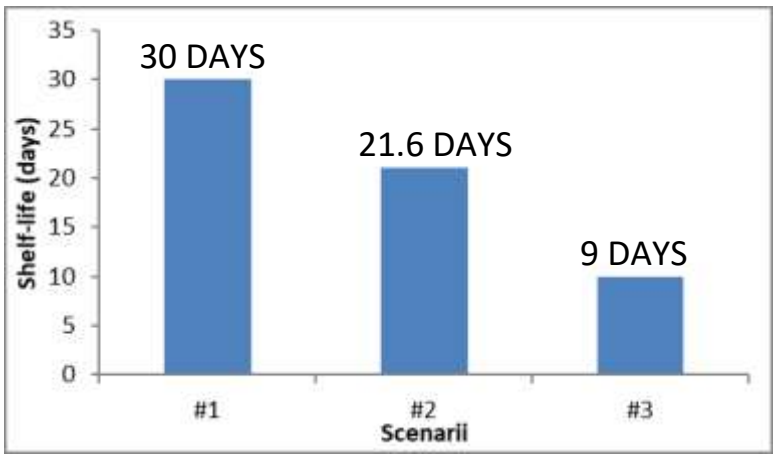


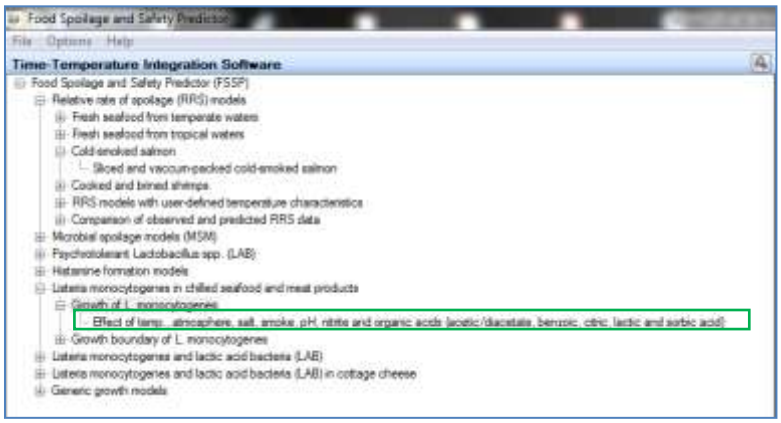
Figure 9: Shelf-life of a meat product related to the different scenarios

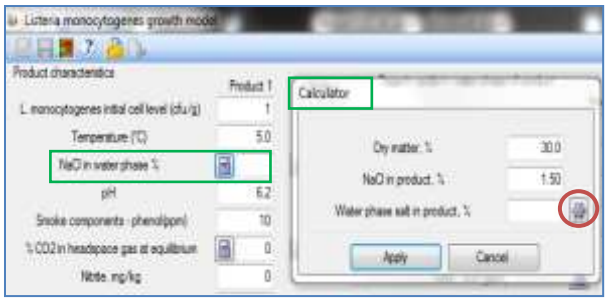
The shelf-life of the product is 30 days in the scenario #1. The shelf-life obtained for scenario #2 and #3 are respectively 1.4 and 3 times shorter.

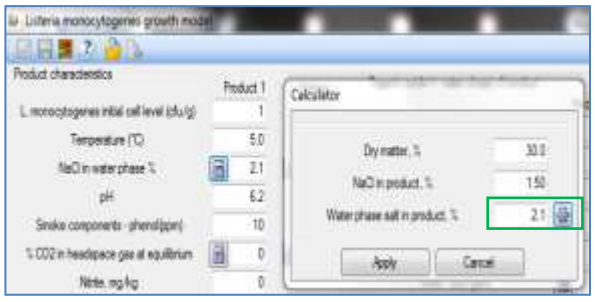
10.10 Use of the FSSP calculator for WPS calculation and a_w calculation

a - WPS calculation

From the FSSP menu, select "*Listeria monocytogenes* in chilled seafood and meat products", then "Growth of *L. monocytogenes*" and "Effect of temp., atmosphere, salt, ...".

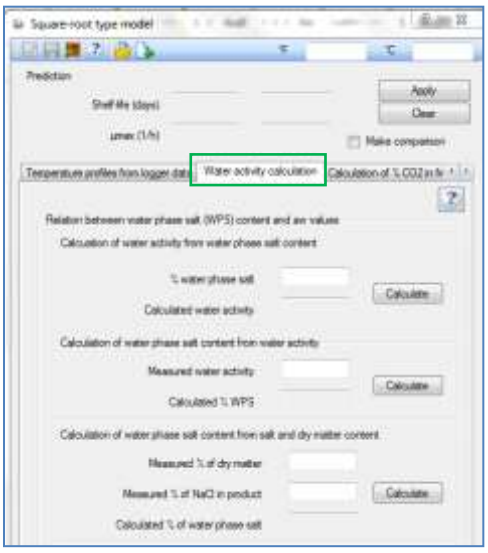






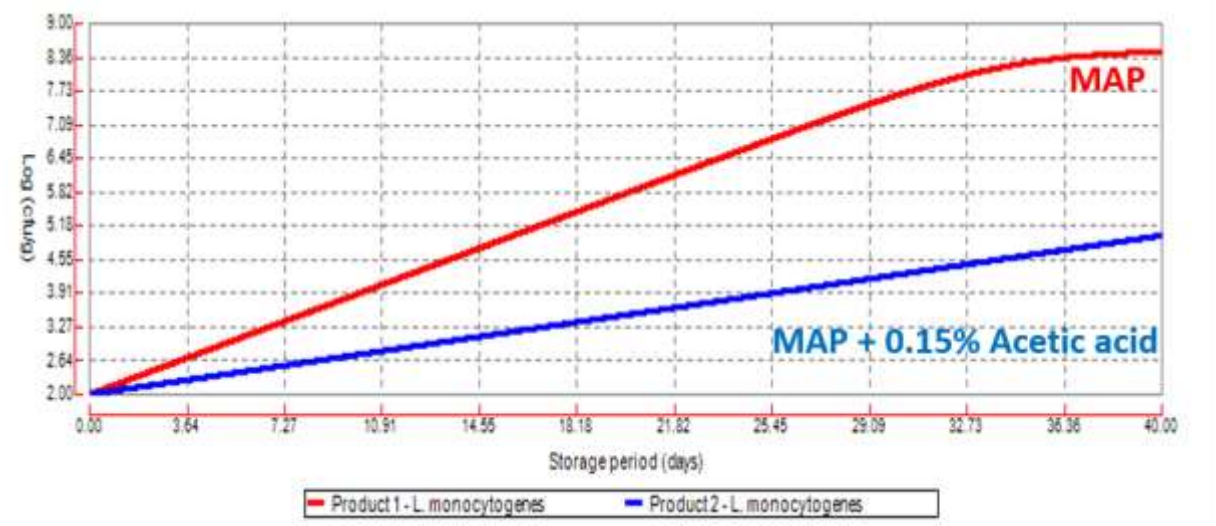
b - a_w calculation

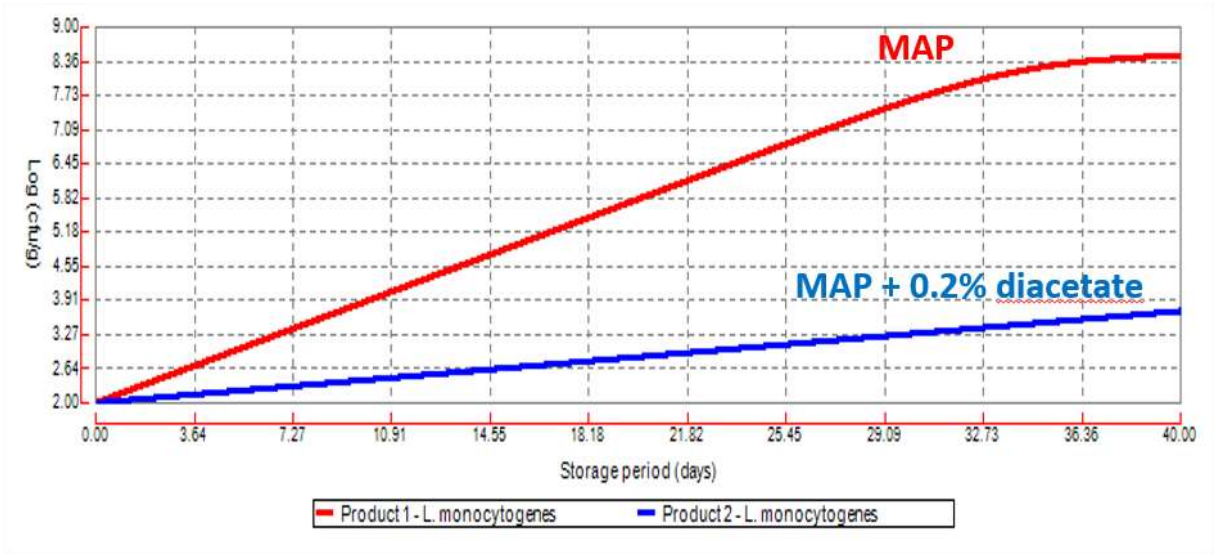
From the FSSP menu, select "Microbial spoilage models (MSM)", then "MS models with user defined parameter values" and "Square-root type model"

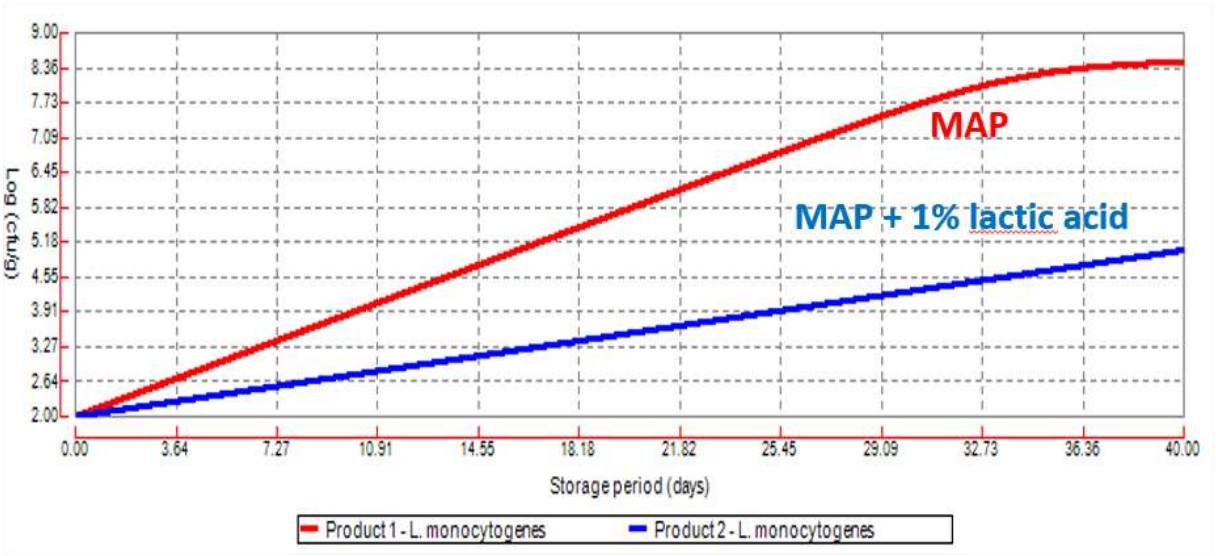


10.11 Examples of the use of organic acids as food preservatives

Lm growth simulations with Food Spoilage and Safety Predictor (FSSP) software on food products under MAP condition with or without organic acids.







10.12 Gas atmosphere measurement to check the tightness of the packaging

Example:

- The concentration of the gas mixture used is 70% N2 and 30% CO2,
- Concentration of the gas mixture in the packaging of the food control samples at t0 and, at end and on the control unit at tend of the challenge test.

Table 13. Example of gas mixture measurement

Control	Time of analyses	batch 1	batch 2	batch 3
Food control sample (without septum)	t_0	0.8% O_2 7.9 %CO_2	0.6%O_2 7.5% CO_2	0.6%O_2 7.9% CO_2
Control unit (with septum)	t_{end}	0% O_2 16.7 %CO_2	0% O_2 22.6% CO_2	0% O_2 13.8% CO_2
Food control sample (without septum)	t_{end}	0% O_2 22.9% CO_2	0% O_2 35.0% CO_2	0% O_2 13.0 %CO_2

At t_{end} , the comparison of the O_2 concentration, on a control unit (with a septum) and on a food control sample (without a septum), informs on the good tightness of the packaging during the test period (concentration equal to zero in all the packaging). The concentration of CO_2, in these controls (control unit and food control sample) can be helpful for the interpretation of *Lm* growth. In case $C0_2$ could also be monitored in test units, this might be useful data in identifying the presence of outliers (in case of high difference in *Lm* growth between units).

10.13 Example of preparation of the initial suspension

The total quantity of the test unit has to be analysed after artificial inoculation.
In case of a large quantity of the test unit, the initial suspension can be prepared by:

- portioning the test units and analysing all the portions, or;
- analysing the entire portion and preparing the initial suspension in 2 steps: performing 2 successive dilutions, for example the 1st dilution in half and then the 2nd dilution at 1/5.

example: the first dilution is made by taking 50 g of the matrix with 50 ml of the diluent. They are mixed and then for the second dilution, 20 g of the first dilution in half are diluted with 80 ml of the diluent.

10.14 Examples on the total number of units required for challenge test assessing a maximum growth rate

Table 14. Example 1: Products under air or vacuum packed – 3 batches analysed

Type of units	Type of analysis	Number of units and date of analyse per batch	
Test units	Enumeration of *L. monocytogenes*	**11**	3 at t_0 and 8 for the growth curve with 5 in the exponential phase
Food control samples	Detection of *L. monocytogenes*	**1**	1 at t_0
	Measurement of physico-chemical characteristics		
	Enumeration of the associated microflora		
Control units	Measurement of physico-chemical characteristics	**2**	1at t_0 and 1 at t_{end}
	Enumeration of the associated microflora		
	Temperature control	**1**	All along the test
Total number required per batch		**15***	

Total number required for 3 batches	**45***

(*) According on the amount of product in the units, this total number might need to be increased in order to have sufficient quantity of product to perform all the required analyses.

Table 15. Example 2: Products under MAP – 3 batches analysed

Type of units	Type of analysis	Number of units and date of analyse per batch	
Test units	Enumeration of *L. monocytogenes*	**11**	3 at t_0 and 8 for the growth curve with 5 in the exponential phase
Food control samples	Detection of *L. monocytogenes*	**2**	1 at t_0 1 at t_0 1 at t_0 1at t_0 and 1 at t_{end}
	Measurement of physico-chemical characteristics		
	Enumeration of the associated microflora		
	MAP measurement		
Control units	Measurement of physico-chemical characteristics	**2**	1at t_0 and 1 at t_{end}
	Enumeration of the associated microflora		
	MAP measurement		
	Temperature control	**1**	all along the test
Total number required per batch		**16***	

Total number required for 3 batches	**48***

(*) According on the amount of product in the units, this total number might need to be increased in order to have sufficient quantity of product to perform all the required analyses.

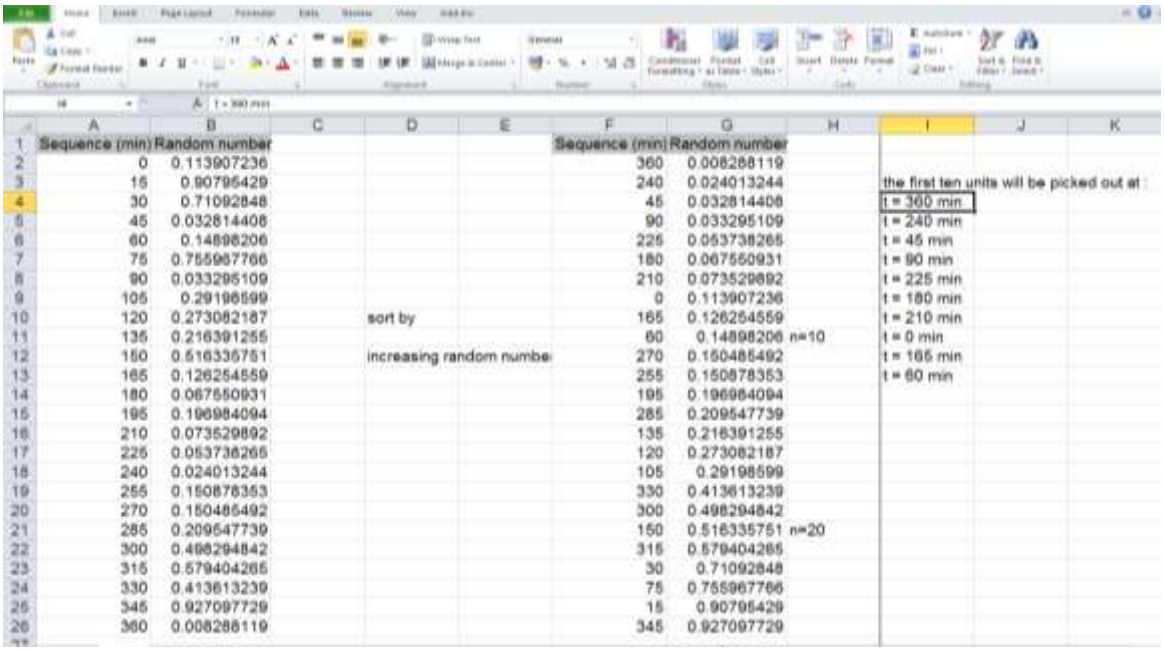
10.15 Single random sampling

A single random sampling method is based on the equiprobability principle. This principle guarantees all the test units of the batch the same chance to be drawn. To satisfy this principle, the size of the batch (N) has to be large enough in comparison to the number (*n*) of test units: n / N < 10%.

One way of achieving a simple random sampling is to number each unit of the batch or in a more practical way the "production time" and then to use random numbers to select the required number of test units. For example, random numbers can be obtained with an Excel sheet with the formula =RAND() (Figure 10), or from random number tables.

Example of a method used to select randomly 10 test units from a batch:

Given that the time for producing one batch is 6 hours, these 6 hours could be divided into periods of 15 minutes. By introducing these sequences in an Excel sheet and using the random function allow to give a random number to each sequence.



	A	B	C	D	E	F	G	H	I	J	K
1	Sequence (min)	Random number				Sequence (min)	Random number				
2	0	0.113907236				360	0.008288119				
3	15	0.90795429				240	0.024013244		the first ten units will be picked out at :		
4	30	0.71092848				45	0.032814408		t = 360 min		
5	45	0.032814408				90	0.033295109		t = 240 min		
6	60	0.14898206				225	0.053738265		t = 45 min		
7	75	0.755967766				180	0.067550931		t = 90 min		
8	90	0.033295109				210	0.073529892		t = 225 min		
9	105	0.29198599				0	0.113907236		t = 180 min		
10	120	0.273082187		sort by		165	0.126254559		t = 210 min		
11	135	0.216391255				60	0.14898206	n=10	t = 0 min		
12	150	0.516335751		increasing random numbe		270	0.150485492		t = 165 min		
13	165	0.126254559				255	0.150978353		t = 60 min		
14	180	0.067550931				195	0.196984094				
15	195	0.196984094				285	0.209547739				
16	210	0.073529892				135	0.216391255				
17	225	0.053738265				120	0.273082187				
18	240	0.024013244				105	0.29198599				
19	255	0.150878353				330	0.413613239				
20	270	0.150485492				300	0.498294842				
21	285	0.209547739				150	0.516335751	n=20			
22	300	0.498294842				315	0.579404265				
23	315	0.579404265				30	0.71092848				
24	330	0.413613239				75	0.755967766				
25	345	0.927097729				15	0.90795429				
26	360	0.008288119				345	0.927097729				

Figure 10: Example of a random sampling scheme with an Excel sheet

These random numbers are then classified by increasing numbers and the first ten ones are selected. The sampling is done by picking out the ten units at the selected times, at the end of the production line.