AGENCE FRANÇAISE
DE SÉCURITÉ SANITAIRE
DES ALIMENTS

EU COMMUNITY REFERENCE LABORATORY FOR
LISTERIA MONOCYTOGENES

Site de
Maisons-Alfort

LABORATOIRE D'ÉTUDES ET DE RECHERCHES SUR LA QUALITÉ DES ALIMENTS ET SUR LES PROCÉDÉS AGROALIMENTAIRES

GUIDANCE DOCUMENT FOR CONDUCTING SHELF-LIFE STUDIES TO DETERMINE COMPLIANCE WITH MICROBIOLOGICAL CRITERIA FOR *LISTERIA MONOCYTOGENES* IN READY-TO-EAT FOODS SET OUT IN EC REGULATION No 2073/2005

VERSION 1 - JANUARY 2008

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0. Introduction

0.1. Foreword

This document was prepared by the EU Community Reference Laboratory (CRL) for *Listeria monocytogenes* with a working group of 8 National Reference Laboratories (NRL's) for *Listeria monocytogenes*:

- National Reference Laboratory for Food Microbiology, University of Liège, Belgium;
- Department of Agriculture & Food Laboratories, (Dairy Science Laboratory), Co Kildare, Ireland;
- Laboratory of the Food and Consumer Product Safety Authority (VWA), Zutphen, The Netherlands;
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Of whose the representatives of the Belgian, Irish, Dutch and Swedish NRLs provided an active contribution.

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

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

The CRL agreed to prepare the guidance document at the first NRL Workshop organized on 2-3 April 2007.

0.2. Scope

The aim of this guidance document is to promote a harmonized implementation of the requirements of Regulation No 2073/2005 for shelf-life studies for monitoring *L. monocytogenes.*

Procedures are described on how :

- To conduct experiments on the shelf-life in naturally contaminated products (referred to as "durability studies");
- To conduct experiments on the shelf-life in artificially contaminated products (referred to as "challenge tests");
- To interpret the results obtained against *L. monocytogenes* criteria in ready-to-eat (RTE) foods defined by Regulation (EC) No 2073/2005.

Thus, the scope of this document is limited to describing procedures for determining growth of *L. monocytogenes* in ready-to-eat foods and interpretation of the results obtained to ensure compliance with the criteria set out in Regulation (EC) No 2073/2005. Durability studies and challenge tests are also used to determine the shelf-life of a product but this use is not taken into consideration in this document.

NRL's are requested to forward this document directly or via their national Competent Authority to the concerned food business operators (FBO's) in their own countries and to assist them in its understanding and implementation.

Note: As indicated in Article 3(2) of the Regulation No 2073/2005, it may not be necessary for each FBO to carry out these experiments in every case (see 0.3).

0.3 Microbiological criteria on *L. monocytogenes* of Regulation (EC) No 2073/2005

Regulation (EC) No 2073/2005 defines microbiological criteria for foodstuffs. Annex I of this regulation defines criteria for *L monocytogenes* for three categories of ready–to-eat (RTE) foods. They are described in Table 1.

Criterion 1.2 consists in fact in two criteria. For the purpose of this document, the criterion of the upper line of food category 1.2 will be designated as "criterion 1.2.a" and the criterion of the lower line of food category 1.2 will be designated as "criterion 1.2.b".

Table 1. List of criteria defined on *Listeria monocytogenes* for ready-to eat-foods (extract from Annex I of the Regulation (EC) No 2073/2005)

Food category	Sampling-plan (1)		Limits (2) m = M	Analytical reference method (3)	Stage where the criterion applies
	n	c			
1.1 RTE foods intended for infants and RTE foods for special medical purposes (4)	10	0	Absence in 25 g	EN/ISO 11290-1	Products placed on the market during their shelf-life
1.2 RTE foods able to support the growth of *L. monocytogenes*, other than those intended for infants and for special medical purposes	5	0	100 cfu/g (5)	EN/ISO 11290-2 (6)	Products placed on the market during their shelf-life
	5	0	Absence in 25 g (7)	EN/ISO 11290-1	Before the food has left the immediate control of the food business operator, who has produced it
1.3 RTE foods unable to support the growth of *L. monocytogenes*, other than those intended for infants and for special medical purposes (4) (8)	5	0	100 cfu/g	EN/ISO 11290-2 (6)	Products placed on the market during their shelf-life

(1) n=number of units comprising the sample, c= number of sample units giving values over m or between m and M
(2) m=M
(3) The most recent edition of the standard shall be used
(4) Regular testing against the criterion is not useful in normal circumstances for the following ready-to–eat foods:
- those which have received heat treatment or other processing effective to eliminate *L. monocytogenes*, when recontamination is not possible after this treatment (for example, products heat treated in their final package)
- fresh, uncut and unprocessed vegetables and fruits, excluding sprouted seeds
- bread, biscuits and similar products
- bottled or packed waters, soft drinks, beer, cider, wine, spirits and similar products
- sugar, honey and confectionery, including cocoa and chocolate products
- live bivalve molluscs.

(5) This criterion applies if the manufacturer is able to demonstrate, to the satisfaction of competent authority that the product will not exceed the limit 100 cfu/g throughout the shelf-life. The operator may fix intermediate limits during the process that should be low enough to guarantee that the limit of 100 cfu/g is not exceed at the end of the shelf-life
(6) 1 ml of inoculum is plated on a Petri dish of 140 mm diameter or on three Petri dishes of 90 mm diameter
(7) This criterion applies to products before they have left the immediate-control of producing food business operator, when he is not able to demonstrate, to the satisfaction of the competent authority, that the product will not exceed the limit of 100 cfu/g throughout the shelf-life
(8) Products with pH≤4.4 or a_w≤0.92, products with pH ≤5.0 and aw ≤0.94, products with a shelf-life of less than five days are automatically considered to belong to this category. Other categories of products can also belong to this category, subject to scientific justification

Article 3(2) of this regulation specifies that: " As necessary, the food business operators responsible for the manufacture of the product shall conduct studies in accordance with Annex II in order to investigate compliance with the criteria throughout the shelf-life. In particular, this applies to ready-to-eat foods that are able to support the growth of *Listeria monocytogenes* and that may pose a *Listeria monocytogenes* risk for public health. Food businesses may collaborate in conducting those studies. Guidelines for conducting those studies may be included in the guides to good practice referred to in Article 7 of Regulation (EC) No 852/2004."

1. Shelf-life studies in naturally contaminated foods (durability studies)

1.1. Scope

Durability studies are performed to evaluate the growth or survival of micro-organisms (for example *Listeria monocytogenes)* in a naturally contaminated food product during the shelf-life, under reasonably foreseeable conditions of distribution, storage and use.

The procedure used to perform the shelf-life study must take into account all factors that may impact on the study such as: the sampling method, the number of units sampled, the storage period before analysis, the storage temperature, the methods used to detect and enumerate *L. monocytogenes.*

This document is not intended to cover shelf-life determination but focuses on the interpretation of results relatively to the criteria of the Regulation (EC) No 2073/2005.

1.2. Food sampling

1.2.1. Introduction

Definitions:

- Population: the field of investigation (for example all the products coming from a production line).
- Unit: element of the population (for example one product).
- Batch: group or set of identifiable products obtained from a given process under practically identical circumstances and produced in a given place within one defined production period.
- Sampling: selection of one or more unit(s).
- Sampled sub-population: the selected unit(s), supposed to be representative of the population.

Note : As the term “sample” can designate either an element or the group of elements randomly selected, it won’t be used in part 2 to avoid confusion.

The sampling procedure, under the responsibility of the FBO, must be representative of the population (to take into account the diversity of the population) and must have a good accuracy, which depends on the size of the sampled sub-population.

The statistical literature describes a large number of theoretical sampling methods, such as the simple random sampling, survey with several degrees, laminated survey... These sampling methods are ideal for statistical purposes but have the disadvantage of being difficult to implement in practice: they require an accurate definition of the whole population, as well as to number all the elements of the population.

1.2.2. The simple random sampling

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

When no information on the structure of the population is available, the most objective way to pick out units is to give all the elements of a population the same chance to be selected. One way of achieving simple random sampling is to number each element and then to use random numbers to select the required sampled sub-population. For example, random numbers can be obtained from an Excel spreadsheet with the formula =RAND( ), or from random number tables.

In order to be representative for the whole production process, samples should be drawn throughout the whole production process. This sampling method should be repeated for different production days/batches (same product, produced under similar conditions) to obtain ≥100 data points (see 1.5.2).

In the frame of this document, the aim of the experiment is in most cases to estimate a proportion, either the proportion of contaminated units among all of them, or the proportion of units greater than 100 cfu/g among the contaminated units. From the sampled sub-population (of size *n*) taken randomly from a population (of size *N*), the estimated proportion is then simply the observed proportion $p = r/n$ (where *r* is the number of "positive" results, and *n* the size).

Example

To estimate the proportion of units greater than 100 cfu/g on a production line of RTE Foods, n = 10 units are picked out randomly. On this sampled sub-population, the enumeration results are (in cfu/g) : 20, <10, <10, 60, 180, 30, 120, <10, 210, 110.
Four out of 10 units have enumeration results greater than 100 cfu/g. The proportion of units (p =r/n or 4/10) greater than 100 cfu/g is then estimated to be 40%.

To calculate the confidence interval associated to the estimation of the proportion, there are two cases:

1. When there are at least 100 observations, including at least 5 "positive" observations, and at least 5 "negative" observations, then a 95% confidence interval is defined by the Normal approximation interval $\left[p \pm 1.96\sqrt{\frac{p(1-p)}{n}} \right]$. A calculator can be used.

2. When there are less than 5 positive results (or less than 5 negative results), or less than 100 observations, then a calculator has to be used.
   Such calculators are available on the internet, from
   http://www.causascientia.org/math_stat/ProportionCI.html or
   http://faculty.vassar.edu/lowry/prop1.html for example. The calculated confidence intervals are usually different from one method to another but are in the same order.
   Using the example above, with r = 4 and n = 10, and confidence levels of 95%, various confidence intervals are obtained, i.e.
   - [17%-69%], [16%-68%] from http://www.causascientia.org/math_stat/ProportionCI.html,
   - [14%-73%] from http://faculty.vassar.edu/lowry/prop1.html,

   but all of them are close to [15%-70%].

With n=10, the confidence interval for the estimated proportion is very large, n has to be increased to reduce it. For example, with r = 40 and n = 100, the 95% confidence interval is reduced to [31%-50%].

1.2.3. Laminated survey

This sampling method divides the population into different strata, with each element of the stratum sharing the same characteristics (same process, same physico-chemical characteristics…). In each stratum the units are randomly sampled in proportion to the size of the stratum.

Example

Estimation of the proportion of contaminated RTE foods greater than 100 cfu/g, after one week of production, taking into account the quantity produced per day.
The number of RTE foods produced during the week is 5 000. There are 5 strata (see Table 2).

Table 2. Example of a laminated survey

	Stratum 1 Monday	stratum 2 Tuesday	stratum 3 Wednesday	stratum 4 Thursday	stratum 5 Friday
Number of RTE produced	1000	2000	1000	500	500
Weight of the stratum W_h	0.2	0.4	0.2	0.1	0.1
Size n	40	80	40	20	20
Number of positive above 100 cfu/g	10	10	5	0	0
Proportion p_h	25%	12.5%	12.5%	0%	0%

The estimated proportion of units above 100 cfu/g is defined by the formula :

$p = \sum_h W_h p_h$, where *h* is the number of the stratum, p the general estimated proportion of the sampled sub-population, W_h the weight of the stratum *h*, and p_h the estimated proportion of units above 100 cfu/g, in each stratum.

Here:

p = 0.2*25% + 0.4*12.5% + 0.2*12.5% = 12.5%

The proportion of units above 100 cfu/g is 12.5%.

The estimated variance of the sampled sub-population is :

Var (p) = $\sum_h W_h{}^2$. ($p_h(1-p_h)/n_h$) and the standard deviation is $\sqrt{\text{var}(p)}$

Here:

Var (p) = (0.2)² x [(0.25*0.75) /8] + (0.4)² x [(0.125*0.875)/ 2] + (0.2)² x (0.125*0.875) = 0.00258

$\sqrt{\text{var}(p)}$ = 0.05

The confidence interval is: C.I. = $p \pm 2\sqrt{\text{var}(\bar{X})}$

Here: C.I = 12.5% ± 2*0.05 C.I = [2.5% - 22.5%]

The 95% confidence interval on the estimation of the proportion of units above 100 cfu/g in this production is [2.5% - 22.5%].

Note: The confidence intervals are applicable in this example, because the size *n* is higher than 100, with 5 "positive" observations, and at least 5 "negative" observations. Otherwise, a calculator such as those presented in 1.2.2. should be used to obtain 95% confidence intervals.

1.3. Storage conditions

1.3.1. Introduction

The cold chain is the term used for the continuity of means successively employed to provide refrigerated preservation of perishable foodstuffs from production to consumption.

This cold chain can be divided in different stages such as processing, transport (during distribution as well as after purchase), storage (cold-room storage, display in retail cabinet, domestic refrigerator).

The storage conditions applied during growth studies must comply with the conditions at which the product is subjected until its final consumption.

In these studies, the units from the sampling plan are stored in refrigerated incubators (with a regulation at ±1°C) at a testing laboratory, at temperatures mimicking the cold chain rupture that can occur during

the shelf-life. The storage temperature of the incubator must be recorded throughout the study.

During the cold chain, foods of animal origin, for example, must be maintained at the temperatures defined by Regulation (EC) No 853/2004[1]. However, it is appropriate to introduce a temperature abuse during the cold chain by using a higher temperature.

Given these conditions, different scenarii can be foreseen.

1.3.2. Information available on the cold chain

Examples

Case 1: *Good knowledge of the cold chain*

If, for a product, the conditions (time and temperature) during transport and storage are well documented (e.g. temperature recorded during all these stages), this data can be used to establish the time/temperature scenarii.

The temperature profiles of the RTE food products are monitored during the total cold chain for storage from manufacturer to the consumer's household. In this cold chain, 5 stages are monitored and can be reproduced at the laboratory (Table 3).

Table 3. Example of information about a cold chain

	stage 1 **transport**	stage 2 **cold room storage**	stage 3 **display in retail**	stage 4 **transport after purchase**	stage 5 **domestic refrigerator storage**
temperature	4°C	5°C	6°C	13°C	8°C
Time	6 hours	24 hours	5 days	1 hour	4 days

Case 2: *Cold chain partly under control (under control for distribution but poor control by the consumer)*

In this case, the product can be stored:

- at 4°C, the temperature fixed by Regulation No 853/2004, for 2/3rd of its estimated shelf-life, and then
- at the abuse temperature of 8°C for 1/3rd of its estimated shelf-life.

For a product with a shelf-life of 25 days, the storage conditions will be 17 days at 4°C and then 8 days at 8°C.

The relative durations, 1/3rd or 2/3rd, come from a French study conducted on the cold chain of RTE

[1] Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin, OJEC of 25/06/2004.

foods. This study pointed out that most often food products remain 2/3rd of their estimated shelf-life in a professional environment (Afssa, 2006).
The choice of the temperatures of 4°C for retail and 8°C for domestic refrigerators is based on the outcome of an investigation of temperatures in European countries (see review in EFSA, 2007).

Case 3: *Lack of information on the cold chain*

When the transport period, the storage period, and the temperature conditions are unknown or not adequately controlled, the following storage conditions can be applied:

- at 4°C, the temperature fixed by the Regulation 853/2004 for 1/3rd of its estimated shelf-life, and then
- at the abuse temperature of 8°C for 2/3rd of its estimated shelf-life.

For a product with a shelf-life of 25 days, the storage conditions will be 8 days at 4°C and then 17 days at 8°C.

1.4. Microbiological analyses

Analysis of the units stored under the storage conditions defined above must be carried out at a minimum at the beginning and at the end of the shelf-life study.

1.4.1. Detection method

According to Annex I of Regulation No 2073/2005 (see Table 1), the reference detection method for *L. monocytogenes* is the Standard method EN ISO 11290-1, amended (ISO, 1996, 2004).

According to Article 5 of the same regulation, alternative methods can be used if they can demonstrate to the satisfaction of the competent authority that these procedures provide at least equivalent guarantees. They must meet the following conditions:

(i) to be validated against the reference method (EN ISO 11290-1), according to the protocol of EN ISO 16140 (ISO, 2003) (or other internationally accepted protocol),

(ii) for the proprietary methods (commercial methods), to be certified by a third party according to the same protocol EN ISO 16140.

Detection must be carried out for all the units at the beginning and at the end of the storage period, in order to determine the positive units.

1.4.2. Enumeration method

According to Annex I of Regulation No 2073/2005 (see Table 1), the reference enumeration method for *L. monocytogenes* is the Standard method EN ISO 11290-2, amended (ISO, 1998, 2004).

According to Article 5 of the same regulation, alternative methods can be used if they can demonstrate to the satisfaction of the competent authority that these procedures provide at least equivalent guarantees. They must meet the following conditions:

(i) to be validated against the reference method (EN ISO 11290-2), according to the protocol of EN ISO 16140 (or other internationally accepted protocol),

(ii) for the proprietary methods (commercial methods), to be certified by a third party according to the same protocol EN ISO 16140.

In addition, an alternative method of conventional microbiology can be applied to lower the limit of enumeration of the micro-organism (colony-count technique-CCT using filtration or Most Probable Number – MPN technique).

The enumeration procedure is performed when units are detected as positive for *L. monocytogenes* at the beginning and/or at the end of the storage conditions, in order to evaluate the growth of *L. monocytogenes* and to determine if the limit of 100 cfu/g exceeds at the end of the shelf-life.

According to the Standard method, it is recommended to lower the limit of enumeration at 10 cfu/g by spreading 1 ml of the initial suspension on a Petri dish of 140 mm diameter or across three Petri dishes of 90 mm diameter containing the specific medium for *Listeria monocytogenes*. Lower counts can be enumerated using the MPN or the CCT with membrane filtration procedures (see before).

1.5. Interpretation of results obtained in durability studies

For durability studies conducted several times on the same product, the results can be pooled and an interpretation taking into account all these results can be made. To be pooled, the products should be the same: manufactured with the same ingredients, with the same process and in the same environmental conditions.

1.5.1. Number of pooled positive results < 100

At least 100 positive results are necessary to conclude on this point. Below this number, there is insufficient information from the durability study to allow the FBO to reach a conclusion.

In absence of other information, the microbiological criterion 1.2.b applies: before the food has left the immediate control of the FBO: *n*=5, *c*=0, *m*=absence in 25g (see Table 1).

1.5.2. Number of pooled positive results ≥ 100

The number of 100 positive units was chosen for the accuracy of the estimation. Indeed, if no unit

is quantified above 100 cfu/g among 100 positive units, the upper limit of the 95% confidence interval on the proportion of units above 100 cfu/g among positive units will be below 5% (see e.g. http://www.causascientia.org/math_stat/ProportionCI.html or http://faculty.vassar.edu/lowry/prop1.html).

Example 1: for pooled durability studies, all enumeration results (≥ 100) were below the limit of 100 cfu/g, the criterion applicable for the product is criterion 1.2.a: products placed on the market during the shelf-life: *n*=5, *c*=0 and *m*=100 cfu/g (see Table 1).

Example 2: from pooled durability studies conducted at the end of the shelf-life, at least 1 unit exceeded the limit of 100 cfu/g, the criterion applicable is criterion 1.2.b: before the food has left the immediate control of the FBO: *n*=5, *c*=0, *m*=absence in 25g (see Table 1).

1.6. Study information

If a FBO asks a laboratory to perform such a study, the following aspects should be a formally clarified between the FBO and the laboratory, before, during, and after the experimental part of the study:

- Identification of the batch tested and its manufacturing date
- The stage at which the durability study is conducted: before placing on the market, on the market during the shelf-life, at the end of the shelf-life
- The expected or previously established shelf-life of the product (decision of the FBO)
- Description of the process
- The list of the ingredients used
- The physico-chemical characteristics of the product
- Data related to the durability study conducted
    - Date of sampling
    - Sampling method and number of units selected
    - Date of storage (beginning)
    - Storage conditions (time – temperature) and recording temperature
    - Detection method
    - Number of units analysed for the detection (at the beginning and at the end)
    - Enumeration method
    - Number of units analysed for the enumeration (at the beginning and at the end)
    - Number of positive units (presence in 25g at the beginning and at the end of the shelf-life)
    - Number of units above the limit 100 cfu/g (at the beginning and/or at the end of the shelf-life)

These details must be listed in the test rapport.

2. Challenge tests

2.1. Scope

A microbiological challenge test is an experiment aiming to assess the growth of a (pathogenic) micro-organism (generally identified as a hazard) in a food by artificially inoculating the specific foodstuff with a known quantity of this target micro-organism followed by determining growth of the micro-organism during storage at given temperatures.

Information may be extracted from the results by:

- assessing the growth potential, either at a constant temperature, or during a time/temperature profile (simulating a realistic cold chain for example). The growth potential is the difference between the $\log_{10}$ after growth and the $\log_{10}$ of the initial concentration.
- or estimating the lag time (lag) or the maximum growth rate (µmax) at a fixed temperature. The lag time is the length of a phase during which the growth of a micro-organism population is null. The lag time depends on physico-chemical characteristics of the foodstuff, temperature of storage, stress of the cells. It is expressed in hours or days. The maximum growth rate (µmax) is the slope of the straight line representing the evolution of the population (expressed in natural logarithm) during the exponential growth phase. The maximum growth rate depends on physico-chemical characteristics and temperature of storage. It is expressed in hours $^{-1}$ or days $^{-1}$.

To conduct challenge tests, at least the following factors must be taken into consideration: the product's characteristics, storage conditions (packaging conditions and storage temperature), the shelf-life of the product, the contamination method (type and number of strains, inoculum level, preparation of inoculum and method of inoculation), duration of the challenge study and sample analyses (enumeration method for *L. monocytogenes*).

2.2. Description of product and manufacturing process

2.2.1. Foodstuff

The product's characteristics must be described. These characteristics include: physico-chemical characteristics (pH, a_w, salt content, preservative concentration etc.), structure and packaging conditions (in air, vacuum, modified atmosphere, etc.).

It is also necessary to get information about any other factor (e.g. associated flora) that may influence the behaviour of *L. monocytogenes* and which should be taken into account in the test. The time/temperature profile for the foodstuff must also be available, i.e. the time/temperature combinations that are expected to be met during processing (the internal storage in the establishment), transportation, storage (possible intermediate storage in the distribution centre, display in retail refrigerators and during storage by the consumer). As possible variation in temperature may occur especially during retail and domestic handling, the temperature conditions in these stages must also be taken into consideration. Besides the shelf-life of the product must also be mentioned.

2.2.2. Manufacturing process

A short description of the manufacturing process must be available. It may be deduced from the HACCP system available in the company and must highlight the key stages of the process where (re)contamination of the foodstuff by *L. monocytogenes* may occur or enabling its multiplication, survival or death.

2.3. Protocol of a challenge test to assess growth potential

In the frame of this part 2, the aim of such challenge tests is mainly to assess if a foodstuff is able to support the (out) growth/survival of *L. moncytogenes* or to estimate the growth potential of this micro-organism under foreseeable conditions.

2.3.1. Number of batches

The number of batches to be tested depends on the variability of the physico-chemical characteristics. When the inter-batch variability is limited, the challenge test can be performed on a single batch. When a noticeable inter-batch variability is reported, at least 3 different batches must be tested, which are representative of the variability (variability of physico-chemical characteristics).

The characterization of the inter-batch variability of the physico-chemical characteristics can be performed with the calculator provided together with this document (see Figure 1). It is based on:

- the physico-chemical characteristics of at least 3 batches;
- the temperature of the test;
- the cardinal values of the species: T_{min} (minimum growth temperature), T_{opt} (optimum growth temperature), pH_{min} (minimum growth pH), pH_{opt} optimum growth pH), a_{wmin} (minimum growth a_w), a_{wopt} (optimum growth a_w). Default values for *L. monocytogenes* are provided in the calculator.

Calculations are introduced in the French Standard NF V01-009 (AFNOR, 2007), based on previous works of Augustin *et al.* (2000, 2005).

However, if other physico-chemical or microbial characteristics may have an impact on the growth of *L. monocytogenes*, they must be taken into consideration.

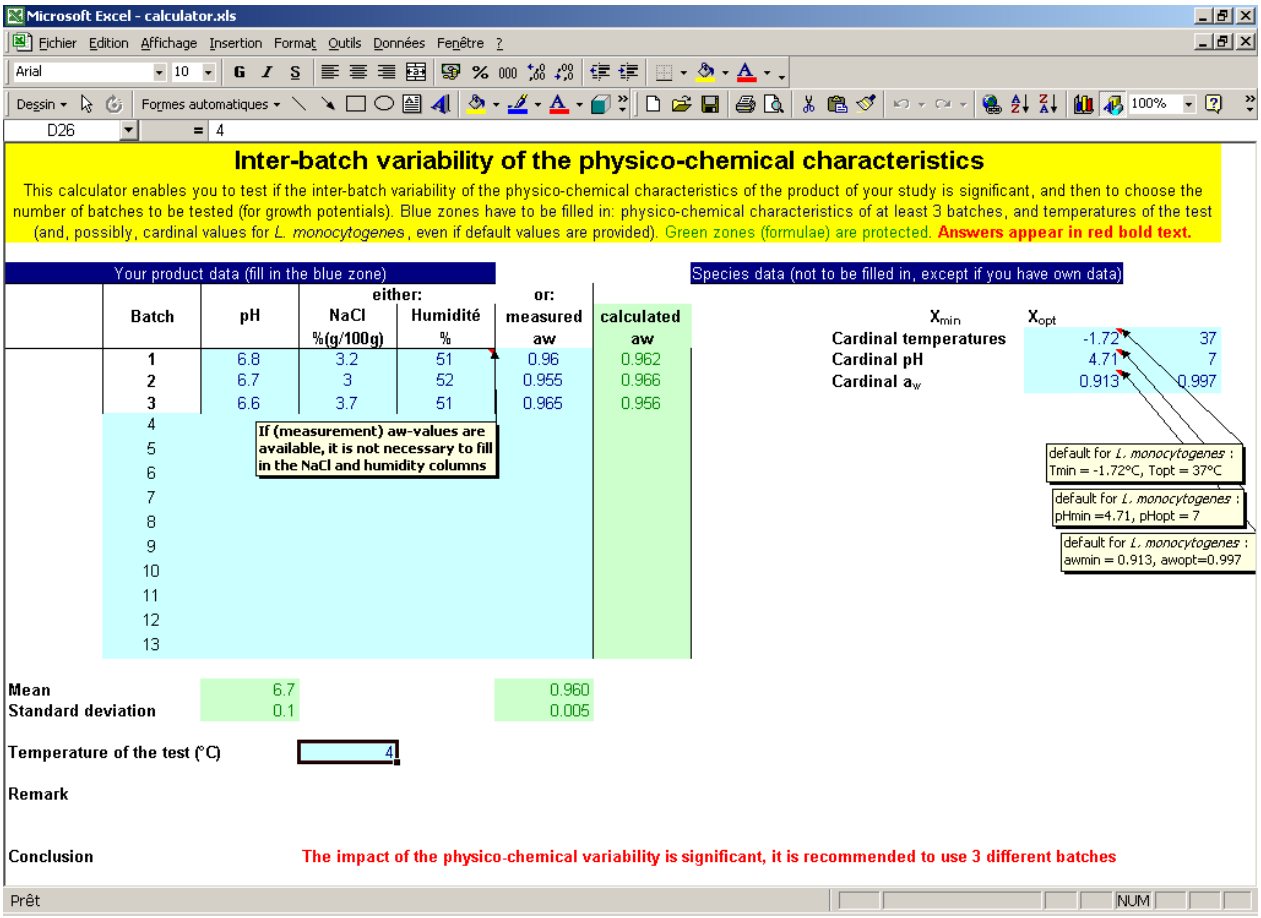


Figure 1. Example of the calculator for the inter-batch variability of the physico-chemical characteristics

Example

Data relating to the species

Default values for *L. monocytogenes*:

T_{min} = -1.72°C T_{opt} = 37°C

pH_{min} = 4.71 pH_{opt} = 7

a_{wmin} = 0.913 a_{wopt} = 0.997

Note that pH_{min} = 4.71 was established with lactic acid. If the main acid in the food is another acid (citric acid, acetic acid), another pH_{min} value may be used.

Data relating to the foodstuff

Batch	pH	a_W
1	6.8	0.960
2	6.7	0.955
3	6.6	0.965

Note that if a_w values are not available, they can be replaced by estimated values, calculated by the calculator on the basis of the WPS (in g/100ml), which is the water phase salt content, i.e. the salt content (in g/100g) divided by the humidity content (in ml/100g) multiplied by 100, with the following equation:

a_w= 1-0.0052471xWPS-0,00012206xWPS^2

Data relating to the test

For example T = 4°C or T= 8°C

Answer (using the calculator provided)

If T = 8°C, the impact of the variability of pH and a_w is not significant, it is sufficient to use one single batch, except if the variability of other physico-chemical or microbial characteristics may have an impact on the growth of *L. monocytogenes*.

If T= 4°C, the impact of the physico-chemical variability is not negligible, it is recommended to use 3 different batches.

2.3.2. Choice of strains

Microbial challenge tests should be performed with a mixture of at least 3-5 strains to account for variations in growth and survival among strains. They are preferably isolated from the foodstuff or a similar food matrix, the production plant and/or from an outbreak in order to account for variation in growth and survival among the strains. It is not necessary to study the chosen strains separately. Prior to the challenge test, the selected strains should at least be tested on growth at the selected temperatures of the test in a medium appropriate for their proliferation (e.g. brain Heart Infusion bouillon).

2.3.3. Preparation of the inoculum

The inoculum level used in a challenge study should be representative of the contamination level expected for the product. Too low inoculum levels may lead to misinterpretation of results. A high inoculum level is not recommended as it may have an impact on natural flora of the product. Thus, an inoculation between 10-100 cfu/ml or cfu/g and preferably as close as possible to the initial contamination in food should be used.

The mixture of strains should contain cells in the stationary phase.

Each strain is subcultured in media at temperature favourable to optimal growth of *Listeria monocytogenes* (Tryptone Soy Broth (TSB), TSB supplemented with Yeast extract (TSB-YE) or Brain Heart Infusion Broth (BHI) at 30^{o}C or 37°C) for a sufficient time to reach the beginning of the stationary phase.

A second subculture is made under identical conditions or under conditions causing a stress (if these conditions are known); different conditions of stress can be applied as shown in the following examples

Examples

Case 1: cold stress

The second subculture is placed at –25°C.

Case 2: osmotic stress

NaCl is added to the second subculture .

Case 3: mineral acid stress

HCl is added to the second subculture.

The exact conditions of stress must be determined in order to reproduce the conditions encountered by the bacteria when the foodstuff is naturally contaminated.

The cultures from each strain are combined (approximately in equal quantity) and mixed before inoculation in the foodstuff.

Successive dilutions are made in a nutrient-free neutral diluent (for example, physiological water) in order to obtain a realistic initial concentration in the foodstuff that means a concentration similar to the initial natural concentration in the foodstuff. The enumeration method has to be adapted to quantify the initial concentration.

If not applying stress conditions to the cells, the individual strains are combined, approximately equal numbers of each strain, and mixed before inoculation into the foodstuff.

The concentration of the inoculum should be checked by enumeration on a selective medium for *Listeria monocytogenes* or on a non selective medium (Tryptone Soy Agar (TSA) for example).

2.3.4. Preparation of test samples

Preparation of samples for physico-chemical characteristics, samples to check the absence of L. monocytogenes

Prior to inoculation, samples must be prepared to determine the physico-chemical characteristics and to check the absence of *L. monocytogenes* in the foodstuff.

The samples used to determine physico-chemical characteristics are inoculated with the free neutral diluent exempted from the bacteria.

Inoculation of samples

The food sample may be an aliquot or a commercial unit of the foodstuff. It can be composed of several parts, of which one being suspected of *Listeria monocytogenes* contamination (e.g. sandwiches).

The inoculation must be as effective as possible at simulating natural contamination conditions and maintaining the intrinsic properties (a_w, pH) of the foodstuff.

The foodstuff or the specific part suspected to be contaminated must be inoculated as follows:

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

In order to minimise changes to the physico-chemical properties, it is recommended that the inoculum does not exceed 1% of the volume of the tested food sample.

The distribution of the inoculum in the food shall mimic the natural distribution of *L. monocytogenes* in the foodstuff: it can be uniform or not.

Packaging of samples

After inoculation, samples must be packaged under the same atmosphere conditions as those that are usually used e.g. air, vacuum or modified-atmosphere packaged.

Minimum number of samples to be prepared

The minimum number of samples to be prepared depends on the heterogeneity of the foodstuff (Table 4). To assess growth potential, 2 sampling times for enumerations may be sufficient:

- "day 0": the time right after inoculation of the product ;
- "day end": the end of the shelf-life.

Table 4. Minimum number of samples to be prepared for one batch (NF V01-009, AFNOR, 2007)

	Homogeneous foodstuff	Heterogeneous foodstuff
Determination of initial concentration of *L. monocytogenes* and associated flora (on day 0)	1	3
Determination of final concentration of *L. monocytogenes* and associated flora (on day end)	1	3
Determination of initial physico-chemical characteristics	1	3
Determination of final physico-chemical characteristics	1	3
Check of the absence of *L. monocytogenes* in the foodstuff prior to the challenge test	3	3
Check of the absence of *L. monocytogenes* in the foodstuff at the end of the challenge test	3	3

2.3.5. Storage conditions for the inoculated foodstuff

See 1.3.

2.3.6. Measurement of physico chemical characteristics

The physico-chemical parameters are measured according to the standard methods.

2.3.7. Enumeration of micro-organisms

L. monocytogenes

See 1.4.2

Associated flora

For enumeration of associated flora a standard method must be used.

2.3.8. Methods for interpreting the enumeration results

During a challenge test on a foodstuff, different measurements at “day 0“ and at “day end“ can be obtained according to the following examples. “Day end” will take into account the foreseeable conditions of use.

Examples

Case 1: the 3 batches are homogeneous – the foodstuff is homogenous

Batch	Day	Concentration (cfu/g)	concentration (log_{10} cfu/g)	Growth potential (log_{10} cfu/g)
1	“day 0“	40	1.60	0.40
	“day end“	100	2.00	

The difference between the counts at “day 0“ and “day end“ does not exceed 0.5 log_{10} . The changes in the count values may be attributed to the measurement uncertainty of the microbiological enumeration, and not to growth of the pathogen. Indeed, according to ISO/TS19036 (ISO, 2006), and to its appendix, the median of the reproducibility standard deviation in solid food products is 0.26 log_{10} (cfu/g), so that the measurement uncertainty *U* is 2x0.26 ≈ 0.5 log_{10}.

These results confirm that the foodstuff cannot support the growth of *L. monocytogenes* according to a standard use and, consequently, the limit of 100 cfu/g may be used from production output and throughout the product’s shelf-life.

The microbiological criterion for this product is criterion 1.3: products placed on the market during their shelf-life: *n*=5, *c*=0, *m*=100 cfu/g (see Table 1).

Case 2: the 3 batches are homogeneous – the foodstuff is heterogeneous

Batch	Day	Concentration (cfu/g)	concentration (log_{10} cfu/g)	Growth potential (log_{10} cfu/g)
1	“day 0“	40	1.60	0.40
	“day end“	100	2.00	
	“day 0“	30	1.48	0.60
	“day end“	120	2.08	
	“day 0“	50	1.70	0.38
	“day end“	120	2.08	

The difference between the counts on “day 0“ and “day end“ exceeds 0.5 log_{10} units once. These results show that the foodstuff can support the growth of *L. monocytogenes* according to a standard use. Then, the criterion is 1.2.a: *n*=5, *c*=0, *m*=100 cfu/g on the market, at the end of the shelf-life (see Table 1). At the producer plant, the intermediate limit is *n*=5, *c*=0, *m*=[2 log_{10}-0.6 log_{10}] = 1.4 log_{10} = 25 cfu/g.

Case 3: the 3 batches are heterogeneous – the foodstuff is homogenous

Batches	Day	Concentration (cfu/g)	concentration (log_{10} cfu/g)	growth potential (log_{10} cfu/g)
1	"day 0"	20	1.30	0.65
	"day end"	90	1.95	
2	"day 0"	15	1.18	0.72
	"day end"	80	1.90	
3	"day 0"	30	1.48	0.41
	"day end"	78	1.89	

The difference between the counts on day 0 and on the "day end" exceeds 0.5 log_{10} units in 2 cases.
These results show that the foodstuff can support the growth of *L. monocytogenes* according to a standard use. Then, the criterion is 1.2.a: *n*=5, *c*=0, *m*=100 cfu/g on the market, at the end of the shelf-life (see Table 1). At the producer plant, the intermediate limit is *n*=5, *c*=0,
m=[2 log_{10}-0.72 log_{10}] = 1.26 log_{10} = 18 cfu/g. For this calculation the higher growth potential has been considered.

Case 4: the 3 batches are heterogeneous – the foodstuff is heterogeneous

Batches	Day	Concentration (cfu/g)	concentration (log_{10} cfu/g)	growth potential (log_{10} cfu/g)
1	"day 0"	40	1.60	0.40
	"day end"	100	2.00	
	"day 0"	30	1.48	0.60
	"day end"	120	2.08	
	"day 0"	50	1.70	0.38
	"day end"	120	2.08	
2	"day 0"	40	1.60	0.25
	"day end"	70	1.85	
	"day 0"	20	1.30	1.18
	"day end"	300	2 .48	
	"day 0"	30	1.48	0.52
	"day end"	250	2.40	
3	"day 0"	40	1.60	0.85
	"day end"	280	2.45	
	"day 0"	80	1.90	0.97
	"day end"	740	2.87	
	"day 0"	70	1.85	0.23
	"day end"	120	2.08	

The difference between the counts on day 0 and on the "day end" exceeds 0.5 log_{10} units in 2 cases.
These results show that the foodstuff can support the growth of *L. monocytogenes* according to a standard use. Then, the criterion is 1.2.a: *n*=5, *c*=0, *m*=100 cfu/g on the market, at the end of the shelf-life (see Table 1). At the producer plant, the intermediate limit is *n*=5, *c*=0,
m=[2 log_{10}-1.18 log_{10}] = 0.82 log_{10} = 7 cfu/g. For this calculation the higher growth potential has been considered.

2.3.9. Study information

If a FBO asks a laboratory to perform such a study, the following aspects should be formally clarified between the FBO and the laboratory (before, during, and after the experimental part of the study):

- All the information necessary for a full identification of the foodstuff tested:
  - Identification of the batch tested and its manufacturing date
  - The recipe (additives, associated flora, etc.)
  - The physico-chemical characteristics of the foodstuff (pH, aw…)

- Data relative to the strains under consideration:
  - Origin of the strains
  - Inoculum preparation conditions, specifically the preparation conditions for the various subcultures and the dilution medium(media) used
  - Inoculum concentration specifying the type of isolation media used.

- Data relative to the actual challenge test:
  - Date of incolulation (starting time)
  - Type of challenge test performed: challenge test for determining the growth potential
  - Mass or volume of the test samples inoculated
  - Inoculum volume and contamination method
  - Sample storage conditions (duration, temperature, hygrometry if necessary) with the curve on whose the storage thermal scenario temperatures are recorded
  - Number of samples analyzed at each enumeration date
  - Reference of the enumeration and detection methods used
  - Mass or volume of the test samples analyzed
  - Quantification threshold of the enumeration method
  - Method used to study the non-contaminated control samples and results obtained
  - Physico-chemical characteristics of the foodstuff at the start and end of the test
  - Associated flora
  - Growth potential

These details must be listed in the test rapport.

2.4. Protocol of a challenge test performed to assess maximum growth rate or lag time

The aim of this challenge test is to obtain a curve with enough sampling times to be able to determine the growth parameters: the lag time (lag), maximum growth rate (μmax) and maximum concentration (Nmax) *for L. monocytogenes*.

2.4.1. Number of batches

See 2.3.1.

2.4.2. Choice of strain(s)

See 2.3.2.

2.4.3. Preparation of the inoculum

See 2.3.3

2.4.4. Preparation of the test samples

Preparation of samples for physico-chemical characteristics, samples to check the absence of L. monocytogenes

See 2.3.4.

Inoculation of the samples

See 2.3.4.

Packaging of the samples

See 2.3.4.

Number of samples to be prepared: example

Two examples of the number of samples to be prepared in the case of a challenge test performed for the estimation of the lag time and the maximal growth rate are shown in the Tables 5 and 6.

Table 5. Example of number of samples to be prepared if the foodstuff is homogenous (for one batch)

	Sampling times	Number of samples
Determination of lag time, µmax, Nmax and associated flora	10 to 15	10 to 15 (1/samling time)
Determination of initial physico-chemical characteristics and associated flora	1	1
Determination of final physico-chemical characteristics	1	1
Check of the absence of *L. monocytogenes* in the foodstuff prior to the challenge test	1	3
Check of the absence of *L. monocytogenes* at the end of the challenge test	1	3

Table 6. Example of number of samples to be prepared if the foodstuff is heterogeneous

	Sampling times	Number of samples
Determination of the lag time, µmax, Nmax and associated flora	10 to 15	30 to 45 (3/sampling time)
Determination of initial physico-chemical characteristics and associated flora	1	3
Determination of final physico-chemical characteristics	1	3
Check of the absence of *L. monocytogenes* in the foodstuff prior to the challenge test	1	3
Check of the absence of *L. monocytogenes* at the end of the challenge test	1	3

2.4.5. Storage conditions for the inoculated foodstuff

The challenge test, in this case, is conducted at a fixed temperature.

The test samples must be stored in an incubator set at the temperature(s) required for the challenge test and within operating margins of ±1°C. The temperature of the storage cabinet must be recorded throughout the challenge test.

2.4.6. Measurement of physico chemical characteristics

See 2.3.6.

2.4.7. Expression of the results

The results of the enumeration are calculated according to the Standard EN ISO 7218 and transformed in base-10 logarithm.

The lag time and the growth rate of each curve (i.e. all the experimental points from one batch) can be easily estimated by non-linear regression. Softwares as MicroFit or Sym'Previus can be used for that purpose.

Example 1: MicroFit software (Figure 2).

This software is based on the Baranyi model as primary model.

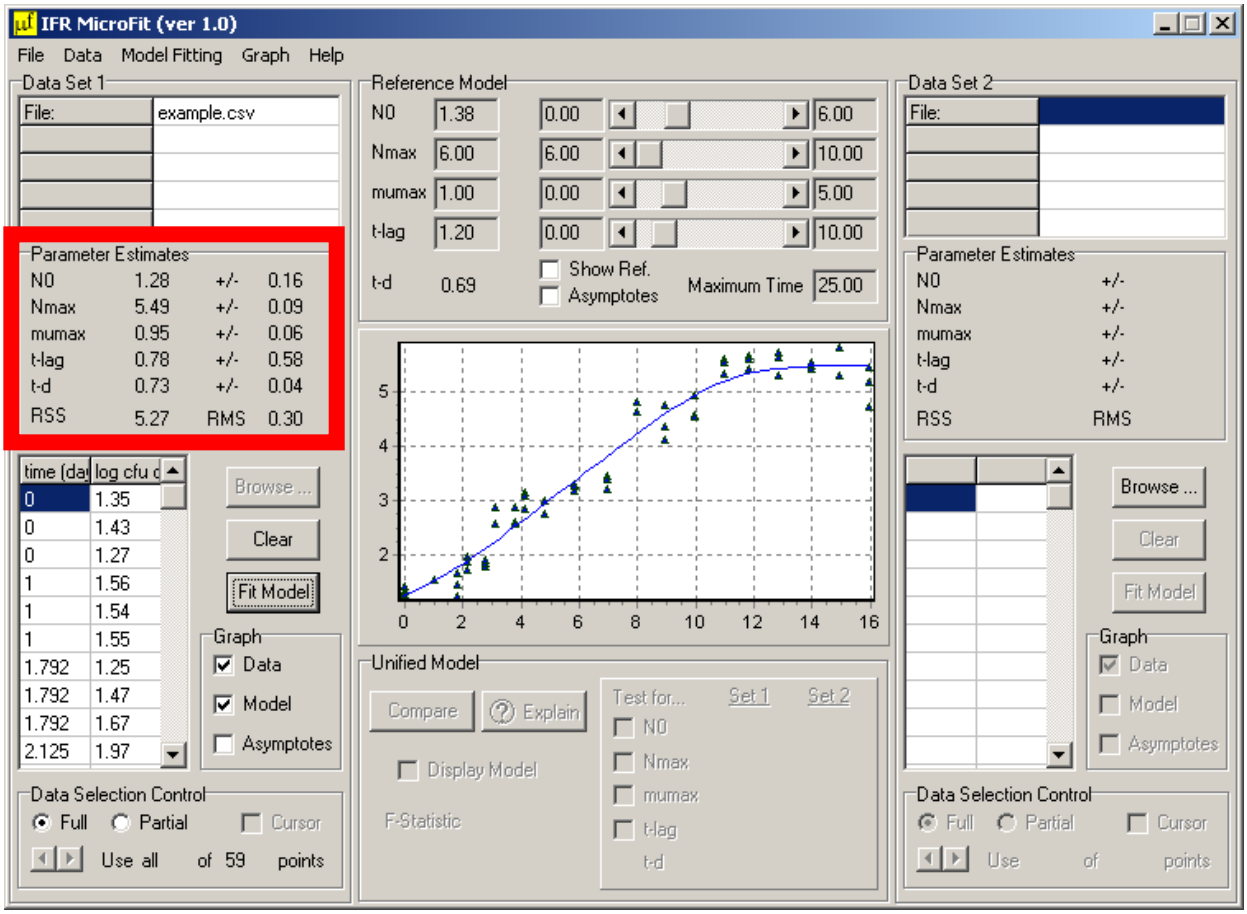


Figure 2. Use of Microfit software to fit a growth curve

The Microfit software provides a chart with the experimental points and the curve fitted by regression, using the Baranyi model. It also extracts the growth parameters of the curve: N0 (logarithm of the initial bacterial concentration , which may also be denoted y_0), Nmax (logarithm of final bacterial concentration, which may also be denoted y_{max} or MPD, for maximal population density), mumax (maximum growth rate, usually denoted μ_{max}), t-lag (lag time, which may also be denoted lag or λ), t-d (generation time or doubling time). This generation time is related to the maximum growth rate by the relationship t-d = $\ln 2/\mu_{max}$. The lag time and generation time are expressed in the time unit (e.g. hours or days) and the maximum growth rate is then expressed in h^{-1} or day^{-1}. Values of parameters are presented with their confidence interval. The software presents also the Residual Sum of Square Root (RSS) and the Root Mean Square (RMS).

Example 2: Sym'Previus (Figure 3)

In this example, the fitting tool of the suiteSym'Previus is used. Same parameters are estimated, but the time unit is hours (and h^{-1} for μ_{max}). The primary model is the lag-logistic model (used by Rosso, 1995), a different one from the Baranyi model used in the Microfit tool (which explains small differences in the estimated results).

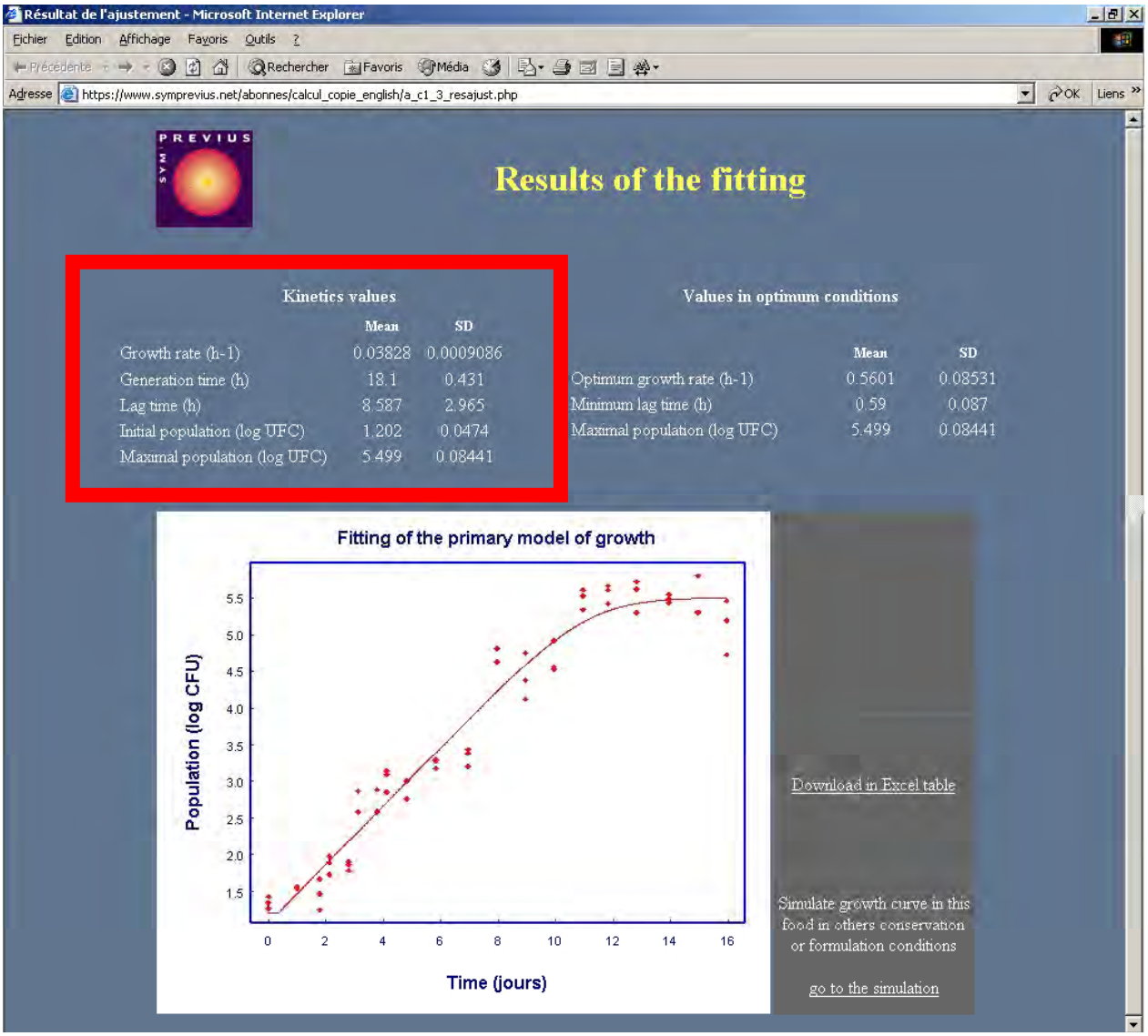


Figure 3. Use of Sym'Previus to fit a growth curve

2.4.8. Methods for interpreting the results

When these results show that the foodstuff can support the growth of *L. monocytogenes* according to a standard use, as in the example above, the criterion is then criterion 1.2a: *n*=5, *c*=0, *m*=100 cfu/g at the end of the shelf-life (see Table 1).

To define an intermediate limit for the end of manufacturing, a growth potential for the whole shelf-life may be used as those described in 2.3.8.

Moreover, additional intermediate limits may be defined during the shelf-life. Thus, the (partial) growth potential for the *d* last days of the shelf-life is: $\frac{\mu_{\max}}{\ln(10)} d$ (due to use of decimal logarithms). It may be replaced by the criterion using the growth potential for the whole shelf-life if less stringent (due to the lag phase).

Using the example above, illustrated by Figures 2 and 3, and assuming a shelf-life of 10 days, the growth potential until the end of the shelf-life is ca. 3.7 $\log_{10}$ (enumeration after 10 days – initial enumeration). Then, the intermediate limit at the end of manufacturing should be:

n=5, *c*=0, *m* = 2-3.7= -1.7$\log_{10}$ cfu/g = 0.02 cfu/g;

As this criterion is more stringent than the default one, the default one can be used:

n=5, *c*=0, *m*=absence in 25g.

Then, along the shelf-life, the following intermediate limits are to be applied:

- At the end of the shelf-life: *n*=5, *c*=0, *m* = 100 cfu/g;
- One day before the end of the shelf-life: *n*=5, *c*=0,

  $m = 2 - \frac{\mu_{max}}{\ln(10)} d = 2 - 0.95/2.3 \times 1 = 1.6$ $\log_{10}$ cfu/g = 40 cfu/g;
- 2 days before the end of the shelf-life: *n*=5, *c*=0,

  $m = 2 - \frac{\mu_{max}}{\ln(10)} d = 2 - 0.95/2.3 \times 2 = 1.2$ $\log_{10}$ cfu/g = 15 cfu/g;
- 3 days before the end of the shelf-life: *n*=5, *c*=0,

  $m = 2 - \frac{\mu_{max}}{\ln(10)} d = 2 - 0.95/2.3 \times 3 = 0.8$ $\log_{10}$ cfu/g = 6 cfu/g;

  …

Similar results are obtained if the estimated results of Sym'Previus (μ_{max} = 0.03828h^{-1} = 0.92 day^{-1}) are used.

2.4.9. Study information

If a FBO asks a laboratory to perform such a study, the following aspects should be formally clarified between the FBO and the laboratory (before, during, and after the experimental part of the study):

- All the information necessary for a full identification of the foodstuff tested:
  - Identification of the batch tested and its manufacturing date
  - The recipe (additives, associated flora, etc.)
  - The physico-chemical characteristics of the foodstuff (pH, $a_{w...}$)

- Data relative to the strains under consideration:
  - Origin of the strains
  - Inoculum preparation conditions, specifically the preparation conditions for the various subcultures and the dilution medium(media) used
  - Inoculum concentration specifying the type of isolation media used.

- Data relative to the actual challenge test:
  - Date the test was performed (start of test)
  - Type of challenge test performed: challenge test for determining the growth potential
  - Mass or volume of the test samples inoculated
  - Inoculum volume and the method of contamination
  - Sample storage conditions (duration, temperature, hygrometry if necessary)
  - Number of samples analyzed at each enumeration date
  - Reference of the enumeration and detection methods used
  - Mass or volume of the test samples analyzed
  - Quantification threshold of the enumeration method
  - Method used to study the non-contaminated control samples and the results obtained
  - Physico-chemical characteristics of the foodstuff at the start and end of the test
  - Monitoring of the gas content within the packaging, if necessary
  - Associated flora
  - A graph showing the experimental results and the curve fitted by regression
  - Lag time and maximum growth rate (with the confidence intervals)

These details must be listed in the test rapport.

3. Interpretation of results according to Regulation (EC) No 2073/2005

3.1. Introduction

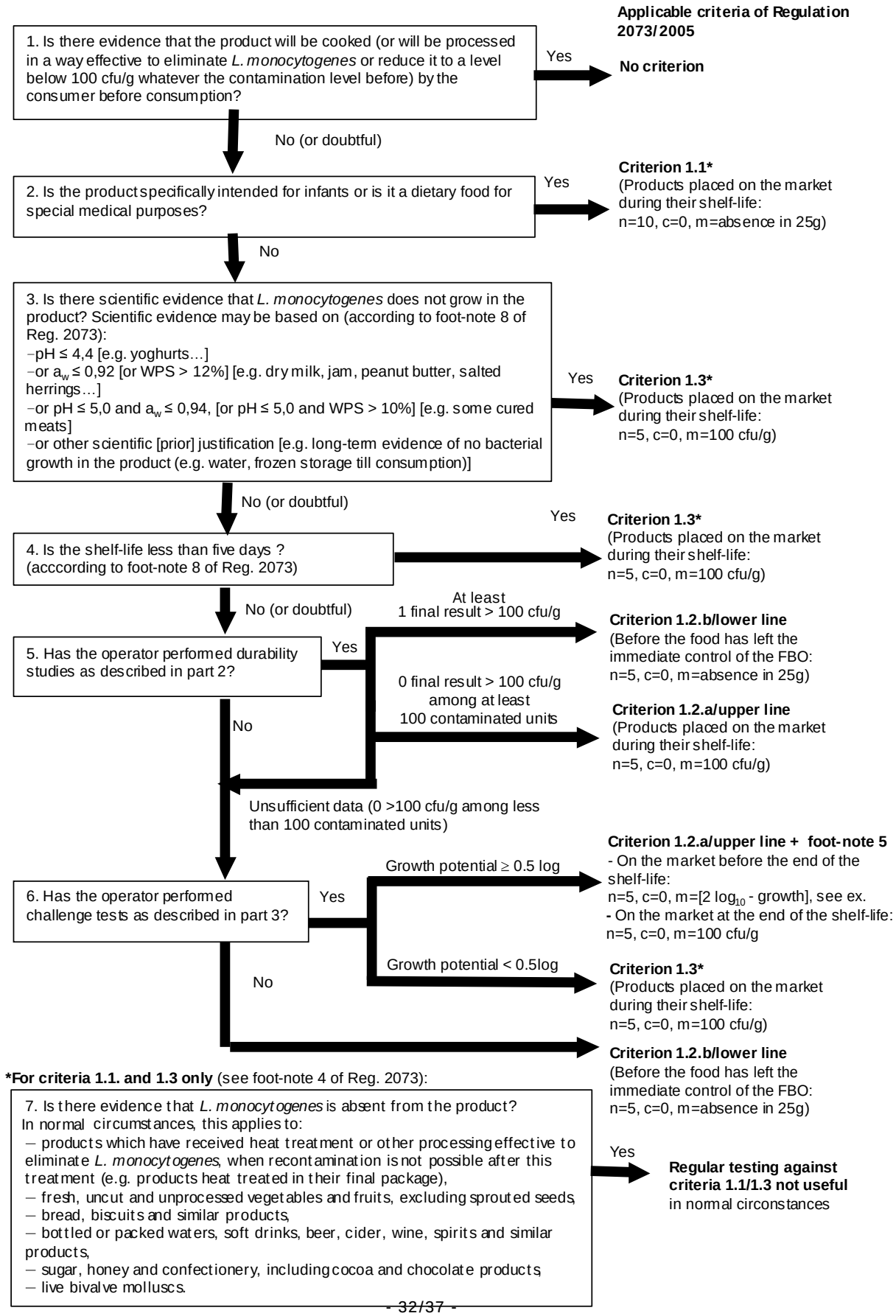
The decision tree (3.2) aims to facilitate interpretation of the results of the studies described in clauses 1 and *2*, in terms of compliance by the FBO's with the microbiological criteria set out in Regulation (EC) No 2073/05 for *L. monocytogenes*. It is only a guidance and it has no regulatory status.

Examples given in clause 3.3. have to be read together with the decision tree, as they illustrate its use.

3.2. Decision tree

See Figure 4.

Figure 4. Decision tree for the criteria to be applied, according to experimental results



3.3. Examples

Examples of implementation of the decision tree (Figure 4) are given below.

◊ **Biscuits labelled "from 8 months":**

1. No (ready-to-eat)
2. Yes (for infants)
   - **Criterion 1.1.: products placed on the market during their shelf-life: *n*=10, *c*=0, *m*=absence in 25g.**

*Yes (biscuits are included in the list of foot-note 4 of the regulation)

- **Regular testing against criterion 1.1. is not necessary**

◊ **Cold-smoked salmon, no challenge test, no durability study:**

1. no (ready to eat)
2. no
3. no
4. no
5. no
6. no
   - **Criterion 1.2.b: before the food has left the immediate control of the FBO: *n*=5, *c*=0, *m*=absence in 25g.**

◊ **Read-smear pasteurised-milk soft cheese, no challenge test, insufficient durability study (0 result above 100 cfu/g, less than 100 results):**

1. no (ready to eat)
2. no
3. no
4. no
5. insufficient data
6. no
   - **Criterion 1.2.b: before the food has left the immediate control of the FBO: *n*=5, *c*=0, *m*=absence in 25g.**

◊ **Spread meat, no durability study, the growth potential (challenge test) in this specific receipt at a realistic fridge temperature is 2 $\log_{10}$ for the whole shelf-life (the growth potential has not been estimated for shorter times):**

1. no (ready to eat)
2. no
3. no
4. no
5. no
6. yes

The criterion is 1.2.a: *n*=5, *c*=0, *m*=100 cfu/g on the market at the end of the shelf-life.

The growth potential is 2 $\log_{10}$ for the total length of the shelf-life. Applying foot-note 5 of the Regulation, **the intermediate limit is "*n*=5, *c*=0, *m*=[2-2] $\log_{10}$ cfu/g= 0 $\log_{10}$ cfu/g=1 cfu/g" before placing on the market**. "*m*=1 cfu/g" can be tested either with a very precise enumeration method (e.g. using a sufficient number of plates, or using filtration).

◊ **Seafood pâté, no durability study, the growth rate (challenge test) in this specific receipt at a realistic fridge temperature is 0.15 $\log_{10}$/day (that is generation time = 2 days), without lag, for a shelf-life of 10 days:**

1. no (ready to eat)
2. no
3. no
4. no
5. no
6. yes

The criterion is 1.2.a: *n*=5, *c*=0, *m*=100 cfu/g on the market at the end of the shelf-life.

The growth potential for 2 days=0.3 $\log_{10}$ (=one doubling). Applying foot-note 5 of the regulation, intermediate limits are fixed:

- "*n*=5, *c*=0, *m*=3 cfu/g" before placing on the market.
- "*n*=5, *c*=0, *m*=6 cfu/g" eight days before the end of the shelf-life.
- "*n*=5, *c*=0, *m*=12 cfu/g" six days before the end of the shelf-life.
- "*n*=5, *c*=0, *m*=25 cfu/g" four days before the end of the shelf-life.
- "*n*=5, *c*=0, *m*=50 cfu/g" two days before the end of the shelf-life.

◊ **Frozen sushi, to be consumed raw after thawing, satisfactory results of durability study with a realistic thawing at room temperature (0 result above 100 cfu/g among 120 naturally contaminated products):**

1. no (ready to eat)
2. no
3. no
4. no
5. yes (0 final result > 100 cfu/g, among at least 100 contaminated units)

➢ **The criterion is 1.2.a: *n*=5, *c*=0, *m*=100 cfu/g, on the market during the shelf-life.**

◊ **Blue-veined cheese (raw milk), the manufacturer demonstrates a growth potential of 0.3 $\log_{10}$**

1. no (ready to eat)
2. no
3. no
4. no
5. no
6. yes (below 0.5)

➢ **The criterion is 1.3: *n*=5, *c*=0, *m*=100 cfu/g, on the market during the shelf-life.**

*: no

◊ **Spread meat, the manufacturers of very similar products (same process, same physico-chemical characteristics) have demonstrated that among 10 000 analysed products at the end of the shelf-life, 100 were contaminated, and all of them were below 100 cfu/g**

1. no (ready to eat)
2. no (recontamination is possible)
3. no
4. no
5. yes

➢ **The criterion is 1.2.a: *n*=5, *c*=0, *m*=100 cfu/g, on the market during the shelf-life.**

◊ **Ice cream:**

1. no
2. no
3. yes (intended for frozen storage till consumption: evidence of no bacterial growth)

➢ **The criterion is 1.3: *n*=5, *c*=0, *m*=100cfu/g.**

*:no

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