anses
French agency for food, environmental and occupational health & safety

EU-RL Lm
European Union Reference Laboratory for *Listeria monocytogenes*

EC REGULATION AND TECHNICAL GUIDANCE DOCUMENT

Hélène BERGIS

EC Regulation No 2073/2005 on microbiological criteria for foodstuffs

General requirements of the EC Regulation

- " Food Business Operators (FBOs) shall ensure that foodstuffs comply with the relevant microbiological criteria set out in Annex I. "

- "…the FBOs responsible for the manufacture of the product shall conduct studies ….. to investigate compliance with the criteria throughout the shelf-life. "

- " In particular, ….for ready-to-eat (RTE) foods that are able to support the growth of *Listeria monocytogenes*…."

EC Regulation No 2073/2005 on microbiological criteria for foodstuffs

- *Listeria monocytogenes* is a concern for **RTE foods** because :
  - ✓ they **can be contaminated** by this bacteria
  - ✓ they **may support the growth** of *L. monocytogenes*
  - ✓ they **will be eaten without cooking** or other processing effective to eliminate or reduce the level of this pathogen

- Annex I of this Regulation lays down microbiological criteria for *L. monocytogenes* in RTE foods

EC Regulation No 2073/2005 on microbiological criteria for foodstuffs

Food safety criteria defined for RTE foods / *L. monocytogenes*

(extract from Annex I of Regulation (EC) No 2073/2005)

Food category	Sampling-plan		Limits m = M	Stage where the criterion applies
	n	c		
1.1 RTE foods intended for infants and RTE foods for special medical purposes	10	0	Absence in 25 g	Products placed on the market during their shelf-life
1.2 RTE foods able to support the growth of *L. monocytogenes*	5	0	100 cfu/g	Products placed on the market during their shelf-life
	5	0	Absence in 25 g	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*	5	0	100 cfu/g	Products placed on the market during their shelf-life

EC Regulation No 2073/2005 on microbiological criteria for foodstuffs

❖ Annex II of this regulation **specifies the studies** that shall be conducted, **when necessary**,
to demonstrate that the products comply with the quantitative criteria for *L. monocytogenes*

- predictive microbiology
- challenge-tests
- durability studies

BUT, Annex II does not describe

- how to choose the appropriate approach and
- how to conduct such studies

EC Regulation No 2073/2005 on microbiological criteria for foodstuffs

To help **FBOs** and **laboratories** to deal with shelf-life studies related to *L. monocytogenes* in RTE foods

2 guidance documents have been produced

- 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

http://ec.europa.eu/food/food/biosafety/salmonella/docs/guidoc_listeria_monocytogenes_en.pdf

- This guidance document intended for FBOs, was released by DG-SANCO
- It helps FBOs to answer to the question:

When and which shelf-life studies are needed?

Technical guidance document on shelf-life studies for *Listeria monocytogenes* in RTE foods

- Technical guidance document on shelf-life studies for *Listeria monocytogenes* in ready-to-eat foods

http://ec.europa.eu/food/food/biosafety/salmonella/docs/shelflife_listeria_monocytogenes_en.pdf

- This guidance document intended for laboratories, was released by the CRL *Listeria monocytogenes*

- It provides 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) 2073/05

- It helps the laboratories to implement:

  - challenge tests assessing a growth potential
  - challenge tests assessing the maximal growth rate
  - durability studies.

Technical guidance document on shelf-life studies for *Listeria monocytogenes* in RTE foods

➢ This guidance document was prepared by the CRL *Listeria monocytogenes* in collaboration with a working group of 8 National Reference Laboratories (NRLs) from:

- Belgium
- Ireland
- Italy
- Poland
- Romania
- Slovakia
- Sweden
- The Netherlands

Technical guidance document on shelf-life studies for *Listeria monocytogenes* in RTE foods

This technical guidance document consists of the following sections:

- Challenge tests assessing a growth potential
- Challenge tests assessing the maximum growth rate
- Durability studies

Technical guidance document on shelf-life studies for *Listeria monocytogenes* in RTE foods

Challenge test to assess the growth potential (δ)

This test aims to answer to 2 questions:

- Is the bacteria able to grow in the considered food?
- If the answer is « YES", what is the range of the growth?

Technical guidance document on shelf-life studies for *Listeria monocytogenes* in RTE foods

Challenge test assessing growth potential (δ)

- Is a laboratory test based on the growth of a bacteria in a food:
  - Artificially contaminated
  - Stored under foreseeable conditions from production to consumption

- Growth potential is calculated according to the formula:

$$\delta = ([L.m] \text{ at the end of the test}) - ([L.m] \text{ at the beginning of the test})$$

- The growth potential can be used:
  1. To determine if a food permits the growth of *L.m*
  2. To set up the concentration of *L.m* **at the end of the shelf-life** according to the concentration at the plant
  3. To set the concentration **at the production** according to the limit of 100 cfu/g at the end of the shelf-life

Technical guidance document on shelf-life studies for *Listeria monocytogenes* in RTE foods

The growth potential (δ) depends on:

- Intrinsic characteristics of the food: pH, NaCl content, a_w, conservatives, associated microflora, structure …
- Extrinsic parameters: storage temperature, gas atmosphere
- The inoculated strain (s) : strains variation
- The physiological state of the bacteria: cold stress, osmotic stress …
- The shelf-life of the food

Technical guidance document on shelf-life studies for *Listeria monocytogenes* in RTE foods

Points to take into consideration

- Product characteristics and shelf life of the product
- Number of batches
- Choice of the strains
- Preparation of the inoculum
- Minimal number of test units per batch
- Inoculation of the test units
- Storage conditions for the inoculated foodstuff
- Measurement of physical-chemical characteristics
- Microbiological analyses: detection and enumeration methods
- Calculation of the growth potential

Technical guidance document on shelf-life studies for *Listeria monocytogenes* in RTE foods

Point 1: product characteristics and shelf-life

Need to collect data from the FBO on the concerned RTE food

- Composition of the RTE food
- Physico-chemical characteristics (pH, aw, …)
- Total microflora and associated microflora
- Packaging condition
- Shelf-life of the RTE food

Technical guidance document on shelf-life studies for *Listeria monocytogenes* in RTE foods

Point 2: number of batches

- At least 3 different batches are tested (variability, representative of the production period)

Point 3: choice of the strains

- The test should be performed with a mixture of at least 3 strains

One of them is a reference strain

The others are isolated from the same food or a similar food

Technical guidance document on shelf-life studies for *Listeria monocytogenes* in RTE foods

Point 4: preparation of the inoculum

- 2 subcultures are made until to reach the early stationary phase

  →The 1st subculture: in optimal condition

  →The 2nd subculture: at a temperature close to the temperature of the product, to adapt the strain to the condition of the product

- The 2nd subcultures of each strain (3) are mixed in equal quantity

- Appropriate dilutions of the mixture of the strains are made in physiological water, to obtain a contamination level between 50 – 100 cfu/g

Technical guidance document on shelf-life studies for *Listeria monocytogenes* in RTE foods

Point 5: minimal number of test units per batch

	Day 0	Day end
Determination of the concentration of *Listeria mono.*	3	3
Determination of the concentration of associated microflora	3	3
Determination of physico-chemical characteristics	3*	3*
Detection/enumeration of *L.m* in blank samples (optional)	3	3

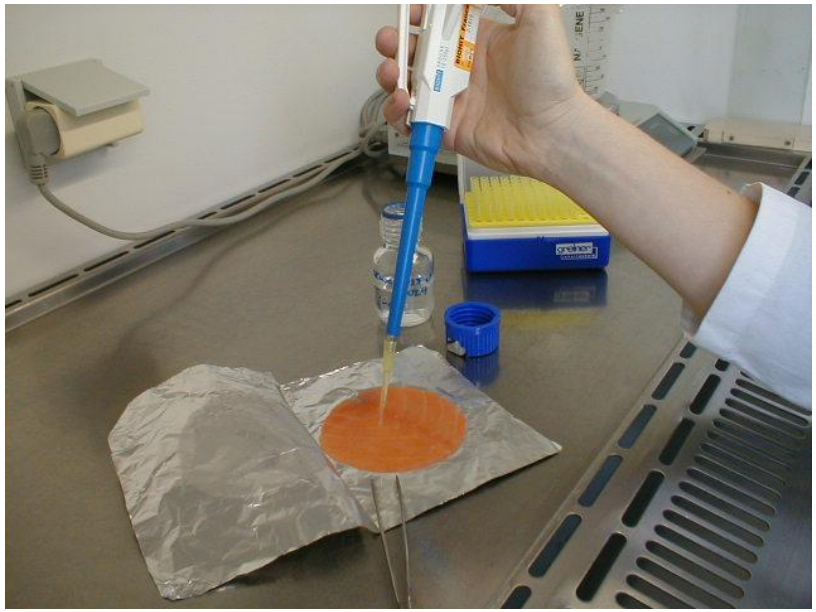
* Only 1 unit, if the product is homogeneous

Technical guidance document on shelf-life studies for *Listeria monocytogenes* in RTE foods

Point 6: inoculation of the test units

- in depth: for food considered homogeneous (ground foodstuff)

- at the surface: to mimic, for example, a contamination during the process of a specific part of the food (e.g. smoked salmon during slicing).



Technical guidance document on shelf-life studies for *Listeria monocytogenes* in RTE foods

Point 7: storage conditions

Reflect the reasonably foreseeable conditions of temperature from production to consumption

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

*Temperature or duration justified by detailed information: the 75th percentile of the observations

Technical guidance document on shelf-life studies for *Listeria monocytogenes* in RTE foods

Point 8: measurement of physico-chemical characteristics

- According to the standard methods

Point 9: microbiological analyses

- For *L. monocytogenes* (detection and enumeration): ISO method or validated methods
- For associated microflora: ISO, CEN, or national standards

Point 10: calculation of the growth potential

For each batch, calculate the difference between the median at day end and the median at day 0.

	Day	Concentration (cfu/g)	Concentration (log_{10} cfu/g)	Difference
1	D0	25	1.40	0.88
		20	1.30	
		55	1.74	
	Dend	100	2.00	
		210	2.33	
		190	2.28	
2	D0	60	1.78	0.84
		30	1.48	
		50	1.70	
	Dend	250	2.40	
		350	2.54	
		390	2.59	
3	D0	20	1.30	0.32
		25	1.40	
		30	1.48	
	Dend	43	1.63	
		52	1.72	
		76	1.88	

Technical guidance document on shelf-life studies for *Listeria monocytogenes* in RTE foods

Point 11: exploitation of the results

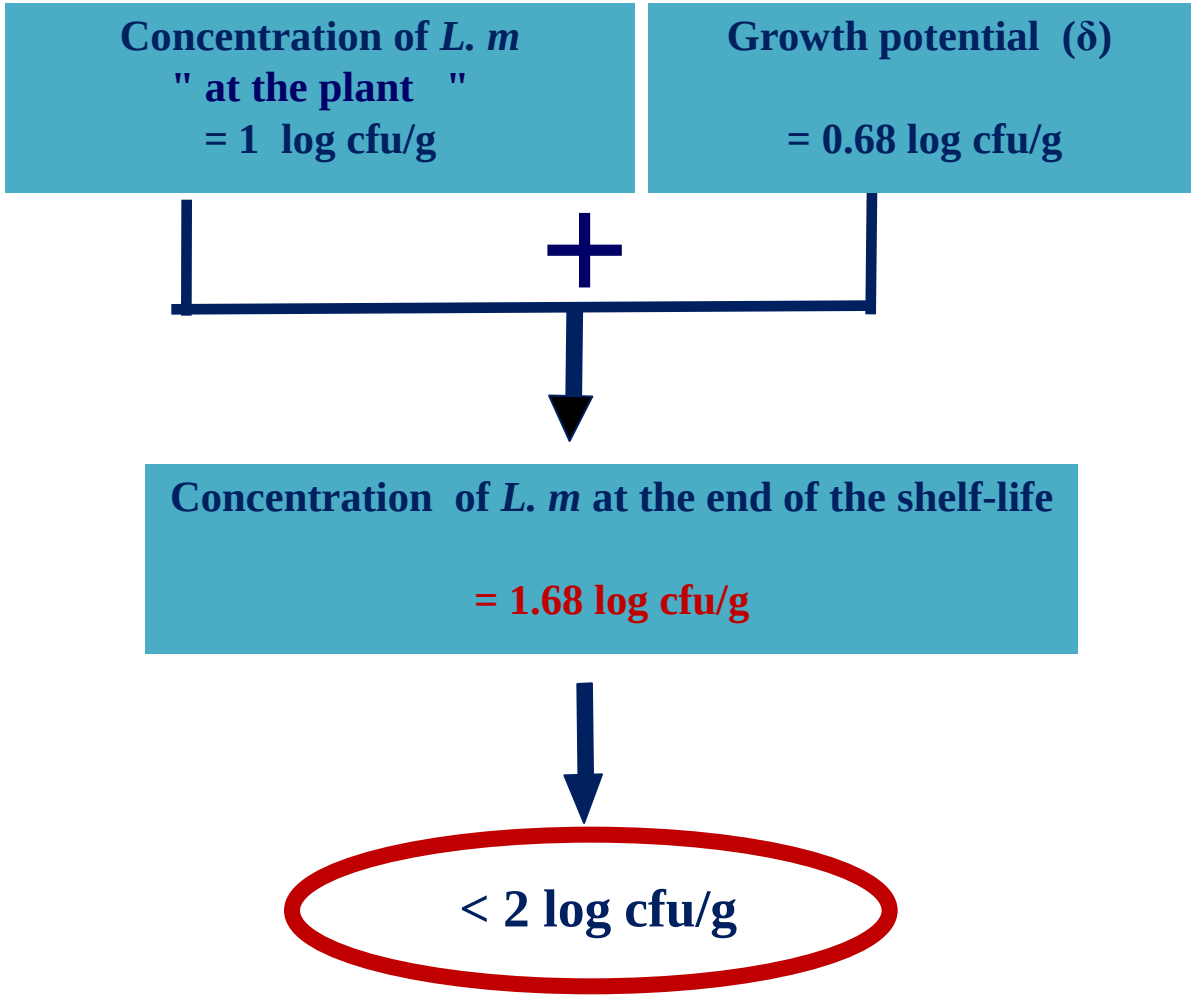
- Is the food able or not to support the growth of *Listeria mono.* ?

  - If $\delta < 0.5$ $\log_{10}$ **cfu/g**, it is assumed that the food is **not able to support the growth of L.monocytogenes**

  - If $\delta \geq 0.5$ $\log_{10}$ **cfu/g**, it is assumed that the food **is able to support the growth of L.monocytogenes**

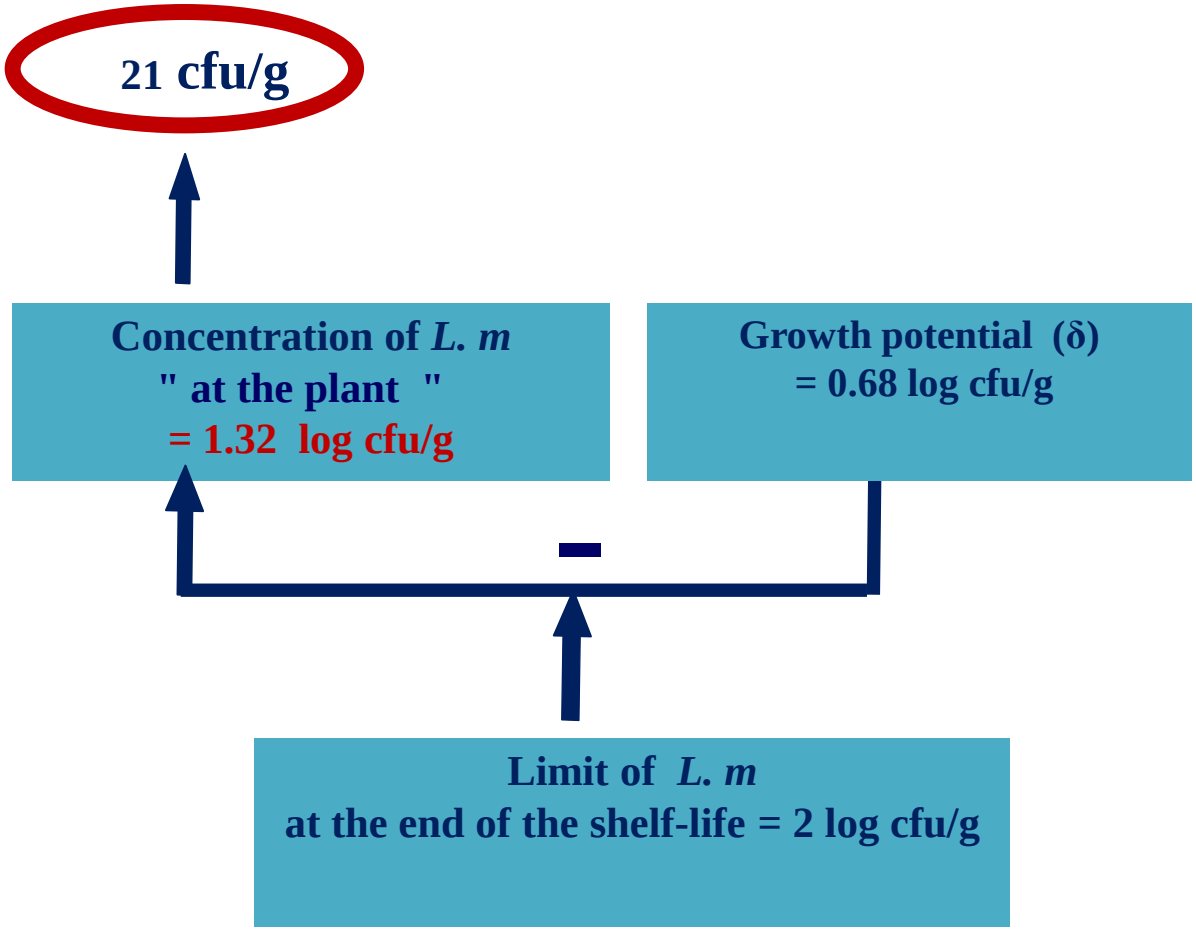
Technical guidance document on shelf-life studies for *Listeria monocytogenes* in RTE foods

❖ **Use of the growth potential: Example 1**



Technical guidance document on shelf-life studies for *Listeria monocytogenes* in RTE foods

❖ **Use of the growth potential: Example 2**



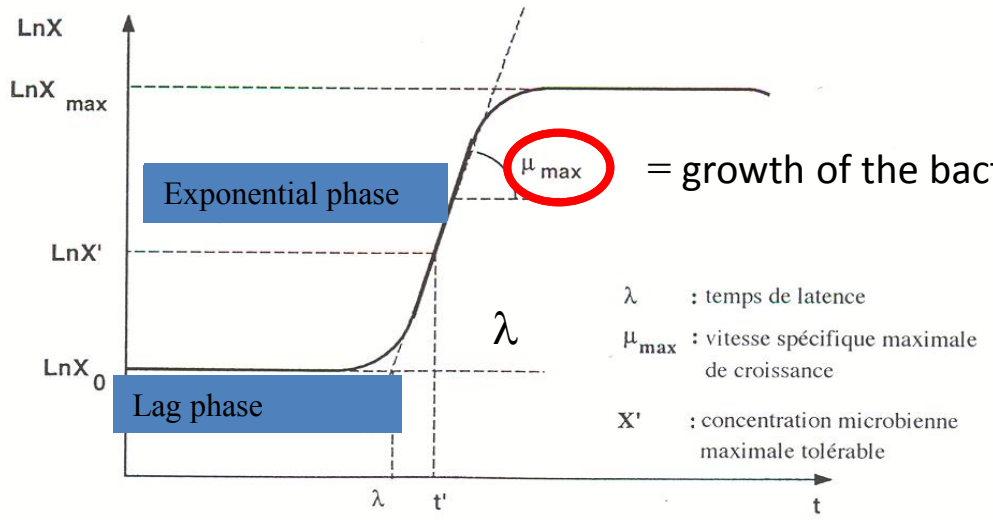
Technical guidance document on shelf-life studies for *Listeria monocytogenes* in RTE foods

Advantages vs disadvantages

- Advantage: it is relatively simple to implement.
- Disadvantage: the exploitation of the result is limited.

Challenge test to assess the maximum growth rate (μ_{max})

- Is a laboratory test based on the growth of a bacteria in a food:
  - Artificially contaminated
  - **Stored at a fixed temperature**



= growth of the bacteria per time unit

Technical guidance document on shelf-life studies for *Listeria monocytogenes* in RTE foods

The maximum growth rate (μ_{max}) depends on:

- Intrinsic characteristics : pH, NaCl content, a_w, conservatives, associated microflora …
- Extrinsic factors: temperature profile, gas atmosphere
- The inoculated strain

Technical guidance document on shelf-life studies for *Listeria monocytogenes* in RTE foods

Points to take into consideration

- Product characteristics
- Number of batches
- Choice of the strains
- Preparation of the inoculum
- Number of test units per batch
- Inoculation of the test units
- Storage conditions for the inoculated foodstuff
- Measurement of physico-chemical characteristics
- Microbiological analyses: detection and enumeration methods
- Calculation of the maximum growth rate

Technical guidance document on shelf-life studies for *Listeria monocytogenes* in RTE foods

Point 3: choice of the strains

- Test each batch with 2 strains, separetely
- 2 fastest strains , among isolates from the same food or a similar food

Point 4: preparation of the inoculum

- 2 subcultures: in optimal condition - until the early stationary phase
- Successive dilutions of 2nd subculture in physiological water, in order to obtain a target level of inoculation about 100 cfu/g

Technical guidance document on shelf-life studies for *Listeria monocytogenes* in RTE foods

Minimal number of test units per batch

	Test units
Determination of the concentration of *L.m*	10 to 15
Detection at "day 0" and enumeration at "day end" of *L.m* in blank samples	3 + 3
Determination of physico-chemical characteristics	3* + 3*
Determination of the concentration of the associated microflora	2 Or 10 to 15

* only 1 unit, if the product is homogeneous

Technical guidance document on shelf-life studies for *Listeria monocytogenes* in RTE foods

Storage condition

- At a fixed temperature, preferably close to the temperature chosen for the prediction

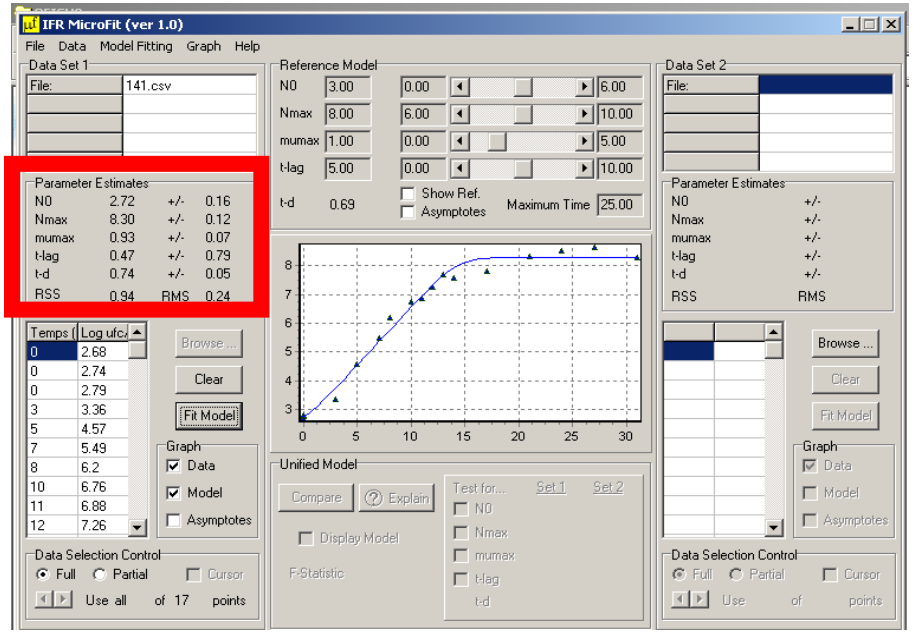
Calculation of the maximum growth rate (μ_{max})

- Results of the enumeration of L. monocytogenes are transformed in $\log_{10}$ cfu/g
- μ_{max} can be estimated from the experimental growth curve by non linear regression

μ_{max}= 0.93 Ln cfu/g * j^{-1}

μ_{max}= 0.40 $\log_{10}$ cfu/g * j^{-1}

at 15°C



MicroFit software

(Baranyi model)

Exploitation of the results

❖ Knowing the value of μ_{max} at a temperature (T_{ref}), it is possible to predict μ_{max} at another temperature (T) in the same food

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

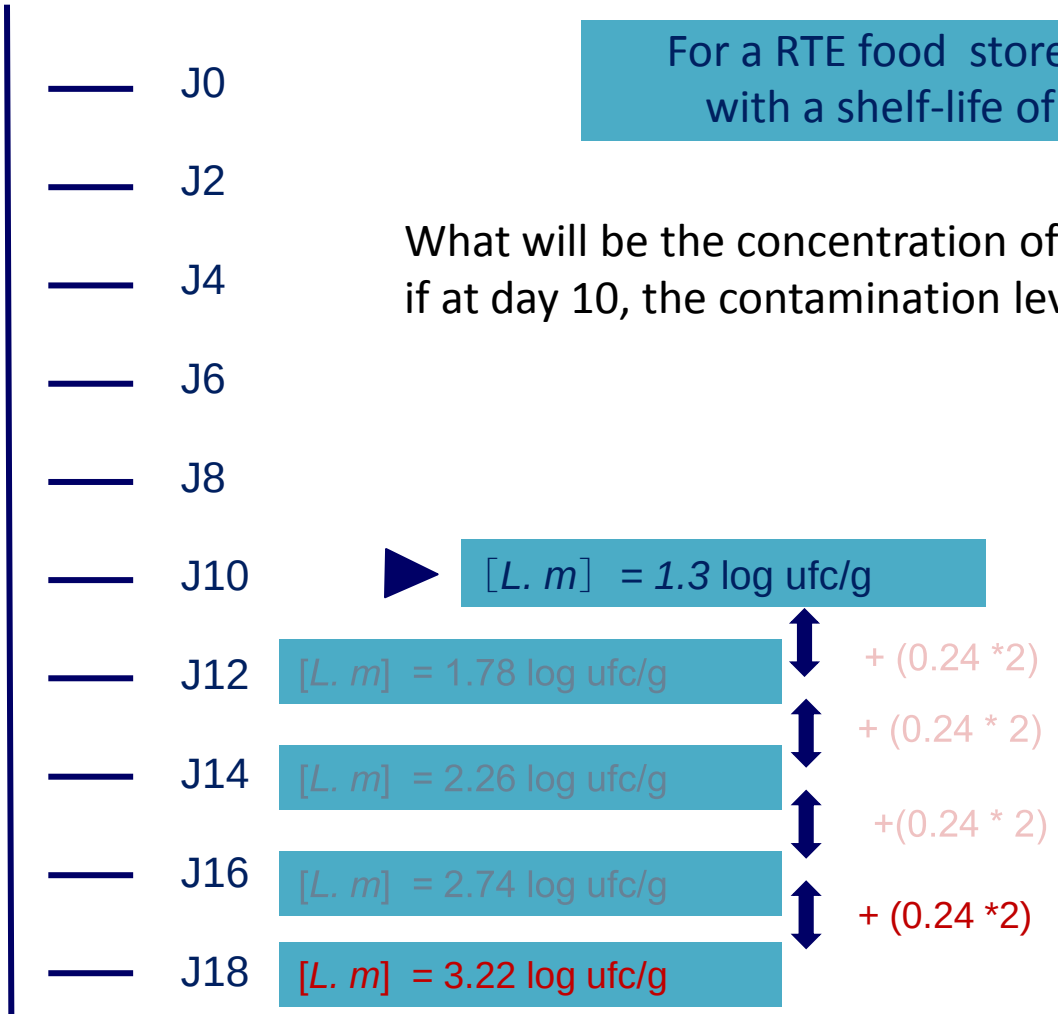
$\mu_{max\,ref}$ = maximum growth rate estimated from the experimental growth curve at T_{ref} with the MicroFit software

T_{min} = minimal growth T° for L.m (- 2°C)

➢ The prediction can be applied to any time-temperature profile

Technical guidance document on shelf-life studies for *Listeria monocytogenes* in RTE foods

❖ Use of the maximum growth rate: Example 1



Technical guidance document on shelf-life studies for *Listeria monocytogenes* in RTE foods

Example 2: Growth of *L. m* during the shelf-life?

- Shelf-life = 12 days,
- Storage conditions = 4 days at 4°C and 8 days at 8°C,

- The estimated daily growth (μ_{max}) at 8°C ⇨ 0.14 $\log_{10}$ cfu/g per day,
- The deduced daily growth (μ_{max}) at 4°C ⇨ 0.05 $\log_{10}$ cfu/g per day.

Growth = 4 times the daily growth at 4°C + 8 times the daily growth at 8°C

Growth = [4 x (0.05)]+ [8 x (0.14)] = 1.32 $\log_{10}$ cfu/g

For the considered product, with such storage conditions, the growth of *L.m*, is estimated to: **1.32 $\log_{10}$ cfu/g**

Advantages vs disadvantages

- **Advantage:**

  - It permits to assess the concentration of *L.m* at any point of the shelf-life.

- **Disadvantages:**

  - It is more expensive, more time-consuming than challenge test for δ.
  - The lag phase and the stationary phase are not included in the calculations.

Technical guidance document on shelf-life studies for *Listeria monocytogenes* in RTE foods

Durability studies

❖ Is a laboratory test based on the growth of a *L.m* in a food:

➔ Naturally contaminated
➔ Stored at foreseeable conditions

It is a end shelf-life own control, following a preservation of samples in reasonably foreseeable conditions

Technical guidance document on shelf-life studies for *Listeria monocytogenes* in RTE foods

How to conduct a durability study ?

Identification of a batch

Sampling

Test units randomly selected

Storage of the selected units **under realistic conditions**

At the end of storage period

Enumeration of *Listeria monocytogenes*

ISO 11290-2 or another validated method

Detection for *L.m* (optional)

ISO 11290-1 or another validated method

Final Result

The result obtained is an estimation of the proportion of units above 100 cfu/g

Sampled units (of size *n*) taken randomly from a batch (of size N)

→ estimated proportion : **p = r / *n***

number of units above 100 cfu/g

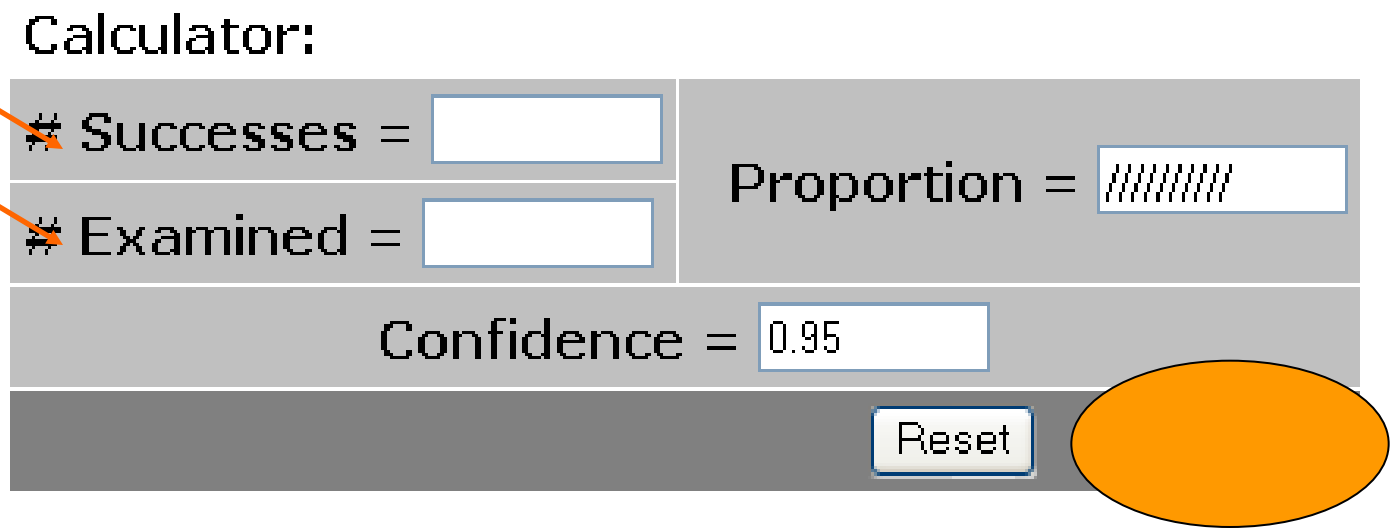
This proportion (p) is associated with **a confidence interval**

It can be obtained by a calculator for example;
http://www.causascientia.org/math_stat/ProportionCI.html

Calculator

Number of units above 100 cfu/g

Number of tested units



Central Confidence Interval:

Lower limit = //////// Upper limit = ////////

Shortest Confidence Interval:

Lower limit = //////// Upper limit = ////////

Technical guidance document on shelf-life studies for *Listeria monocytogenes* in RTE foods

Confidence interval

n	r	p	CI
20	0	0%	[0% - 16%]
100		0%	[0% - 3.5%]
20	1	5%	[1% - 24%]
100		1%	[0.2% - 5%]
20	2	10%	[3% - 30%]
100		2%	[0.6% - 7%]

The **more units** that are analysed, the **narrower the confidence interval**

→ex: the upper limit of the confidence interval for "0 units exceeding 100 cfu/g out of 100 units" is lower than that obtained for "0 units exceeding 100 cfu/g out of 20 units".

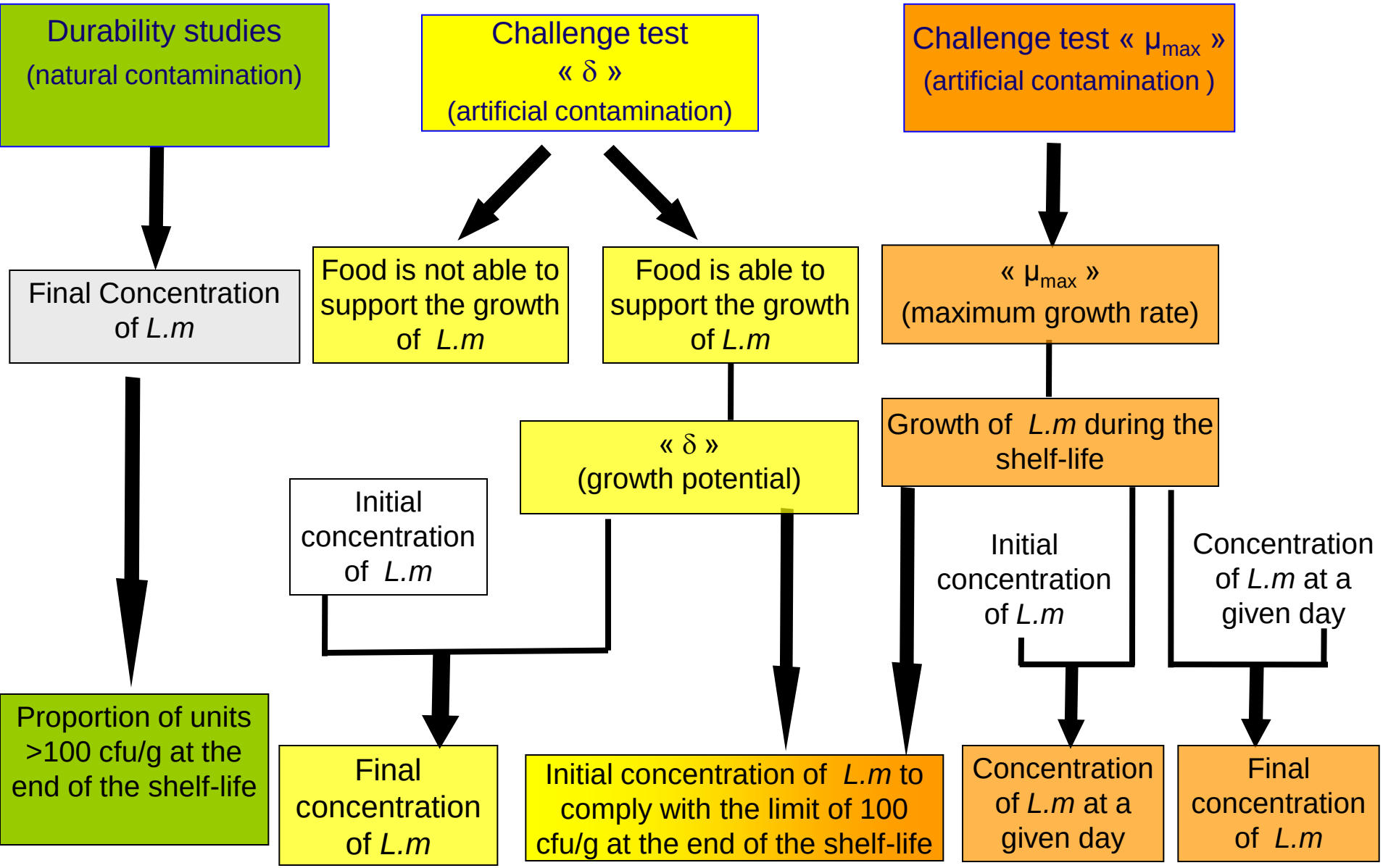
To get a large number of analysed units: to **gather results** of repeated durability studies, performed for one RTE food obtained from the same process.

Advantages vs disadvantages

- **Advantage:** it is more realistic than challenge tests, as the contamination is natural.

- **Disadvantage:** the interpretation of the result may be difficult if the prevalence of the bacteria is low.

Data obtained at the end of these tests?



How to get confidence in the skill of a laboratory implementing CT assessing the growth potential of *L. m*?

- EU RL for *L. m* has written a document listing the points to be checked to be sure that CT assessing growth potential are implemented according to the technical guidance document.

- The document is named:
"Guidance document to evaluate laboratories implementing challenge tests on the growth potential of *L. m* in ready-to-eat foods "

- It was written in collaboration with representatives of 12 NRLs for *L. m* from Belgium, Cyprus, Czech Republic, Denmark, Ireland, Latvia, Norway, Portugal, Slovenia, Spain, Sweden, The Netherlands.

- It will be released by the end of the year.

Objective of the supporting document

- To procure standard examination of laboratories performing challenge tests assessing the growth potential.

- Points of the guidance document:
  - General information related to the laboratory and the FBO
  - Review of data provided by FBO
  - Assessment of the technical skill of the laboratory conducting CT
  - Use of the results
  - Test report
  - Conclusion

Who will use the "supporting document"?

- Either experts basing their opinion on documents.

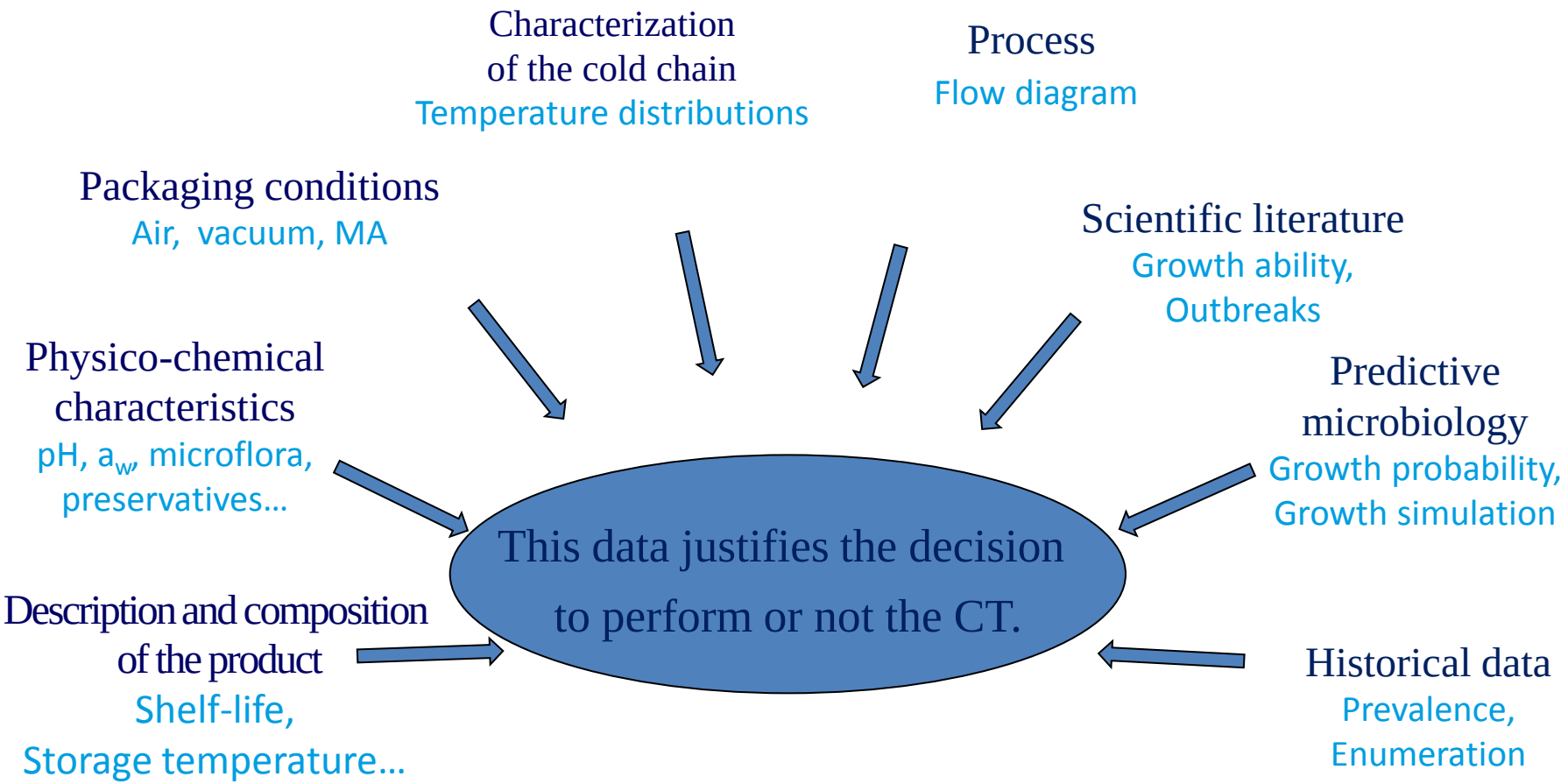
- Or auditors conducting audits in laboratories.

What is the quality requirement for the laboratory?

- The laboratory has to be accredited according to ISO 17025, for the detection and enumeration of *L. monocytogenes*.

- The laboratory has to get satisfactory results from PT trials for parameters such as pH, a_w, preservatives, microflora,…

The expert conducts a review of the data provided by the FBO



This data will help the laboratory to perform the CT.

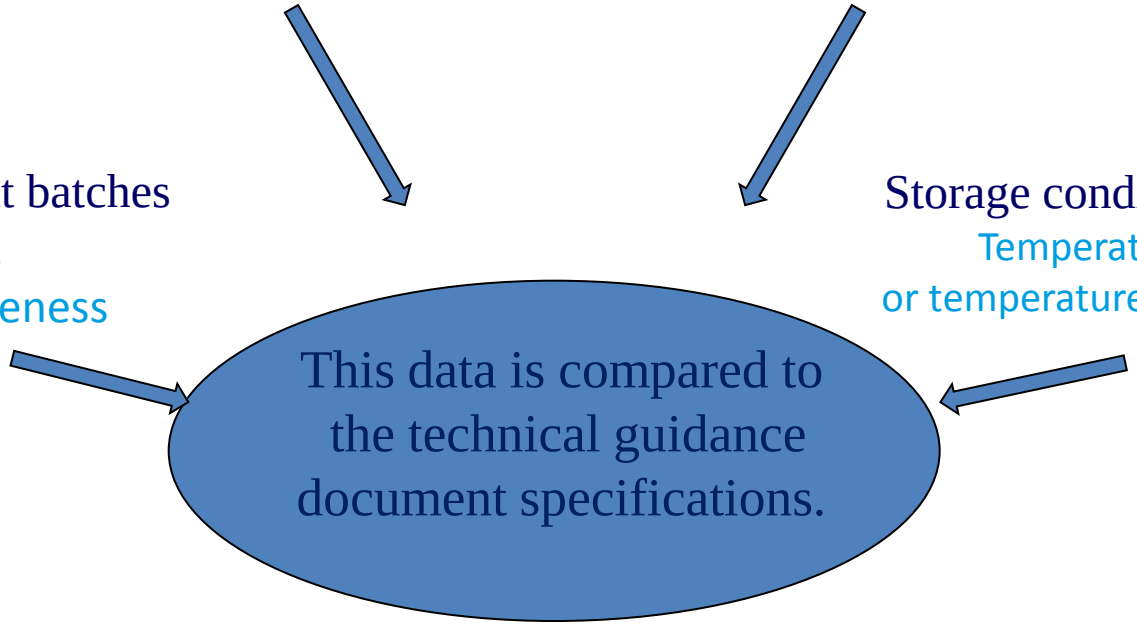
The expert conducts a review of the " setting up " of the CT

Preparation of the inoculum
Number/ source of the strains,
Preparation of the subcultures and inoculum

Inoculation of the samples
Part to be contaminated,
Method and level of inoculation of inoculation

Information about batches
Number,
Representativeness

Storage conditions of the samples
Temperature distributions,
or temperatures proposed by the EU



The expert conducts a review of the results, their use and the test report

Calculation of the growth potential

Concentration of *L. m* at D_0 and D_{end},

Calculation of the growth potential

Enumerations of *L. m* at D_0 and at D_{end} in the inoculated samples

Enumeration method,

Enumeration threshold

Use of the results

Classification of the RTE food,

Calculation of the [*L. m*] at the plant

Characterization of the RTE food

Physico-chemical characteristics,

Enumeration of associated flora,

Blank L. m detection/concentration

This data is compared to the technical guidance document specifications.

Test report

Report number,

Accreditation logo,

Data related to the food

Data related to the CT

What is the final conclusion of the expert (or the auditor)?

- The laboratory is able to perform challenge tests assessing the growth potential

YES □ NO □

- The laboratory will be able to perform challenge tests provided that improvements are made on few points:

1.
2.
3.
4.
5.

THANK YOU FOR YOUR ATTENTION