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

EURL Lm
European Union Reference Laboratory for *Listeria monocytogenes*

EURL *Lm* Technical Guidance Document for conducting shelf-life studies on *Listeria monocytogenes* in ready-to-eat foods (2014)

Annie Beaufort
LSAl, EURL Lm, Mission MOD/AQR

Guidance prepared by the EURL for *Listeria monocytogenes* with a WG:

- 9 National Reference Laboratories (NRLs) coming from: Belgium, Cyprus, Denmark, Ireland, Italy, The Netherlands, Norway, Slovakia, Spain,
- 1 associated NRL (NL)
- 1 laboratory representing a NRL (UK)

Main steps related to the revision

- This version has replaced the second version of November 2008.
- Three meetings were held for discussing the new document:
    - In October 2012 (In Maisons-Alfort),
    - In April 2013 (In Maisons-Alfort),
    - In September 2013 (A teleconference).
- The draft have been presented and agreed by DG SANCO-CA Working Group on microbiological criteria (19/03/2014) then to SCoFCAH (16/06/2014).

What is new in this 3rd version of the technical guidance document ?

• The introduction has been extented.

• In the chapter dedicated to « challenge tests », this third version develops a clause related to « Review of data ».

• Concerning the protocol, several changes have been made in order:
  - to take into account the variability of the batches and the strains,
  - to better ensure food safety as regards to *Lm* risk,
  - to take into account comments from DG SANCO.

• Eight annexes have been added for a better understanding of the guidance document.

Contents

1. Introduction

The introduction is composed of 4 items:

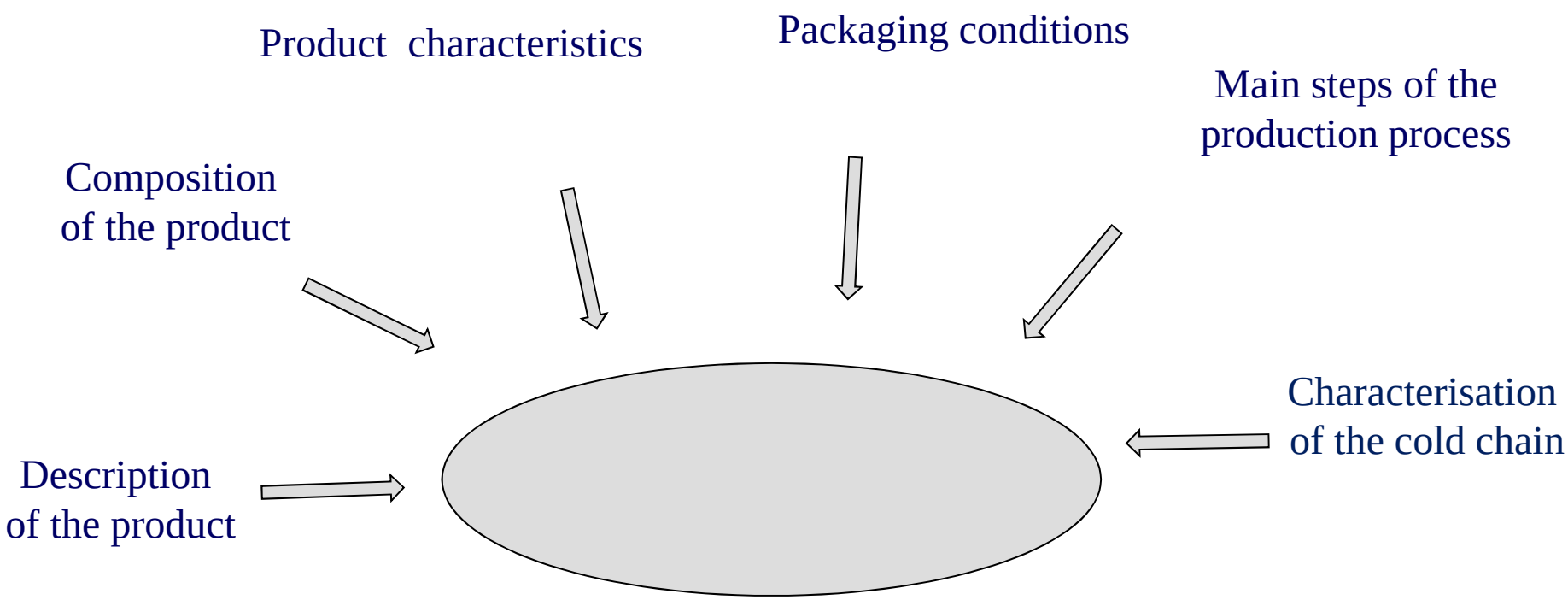
- *Listeria monoytogenes*
- Ready-To-Eat foods
- Legislative background
- EU guidance document dedicated to food business operators

3. Challenge tests

Items covered:

- Review of data
- Challenge tests
  - Challenge test assessing growth potential (δ)
  - Challenge test assessing maximum growth rate ($Rate_{max)}$

Review of data



This data will help to decide if a challenge test is necessary or not.

If yes, it will also enables the laboratory to design the challenge test.

Challenge test assessing the growth potential

- Laboratory test used for the estimation of 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:

δ = ([*Lm*] at the end of the test) – ([*Lm*] at the beginning of the test)

- The growth potential can be used:
  - To classify a food in the category 1.2 or 1.3 of the regulation No 2073/2005:

If $\delta > 0.5$ log cfu/g, it is assumed that the food supports the growth of *Lm*.

If $\delta \leq 0.5$ log cfu/g, it is assumed that the food does not support the growth of *Lm*.

- The growth potential can be also used to quantify the growth of *Lm*.

Challenge tests assessing the maximum growth rate

- Laboratory test used to assess the growth rate of a bacteria in a food:
    - Artificially contaminated,
    - Stored at a fixed temperature.

- This test allows an estimation of the concentration of *Lm* at any day of the shelf-life.

- The advantage of this test is related to its flexibility: knowing μ_{max} at a reference temperature, it is possible to deduce μ_{max} at another temperature using a mathematical formula.

$$\text{Rate}_{max} \text{ at T} = \text{Raftered} \times \frac{(T - Tmin)^2}{(Tref - Tmin)^2}$$

Protocol

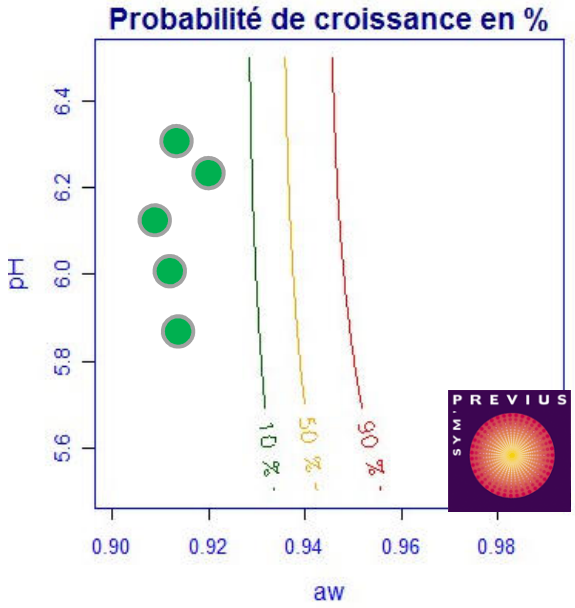
The main items considered:

- Number of batches
- Number of days of analysis
- Number of test units per day of analysis
- Choice of the strains
- Preparation of the test units
- Inoculation
- Storage conditions
- Microbiological analyses
- Calculation of the growth potential or maximum growth rate
- Exploitation of the results

Number of batches to test

- To take into account the variability of the batches, at least 3 batches are tested.
- But, one batch may be enough if the growth probability is low: ≤ 10%.
- It is possible to get the growth probability using a growth/no growth boundary module.

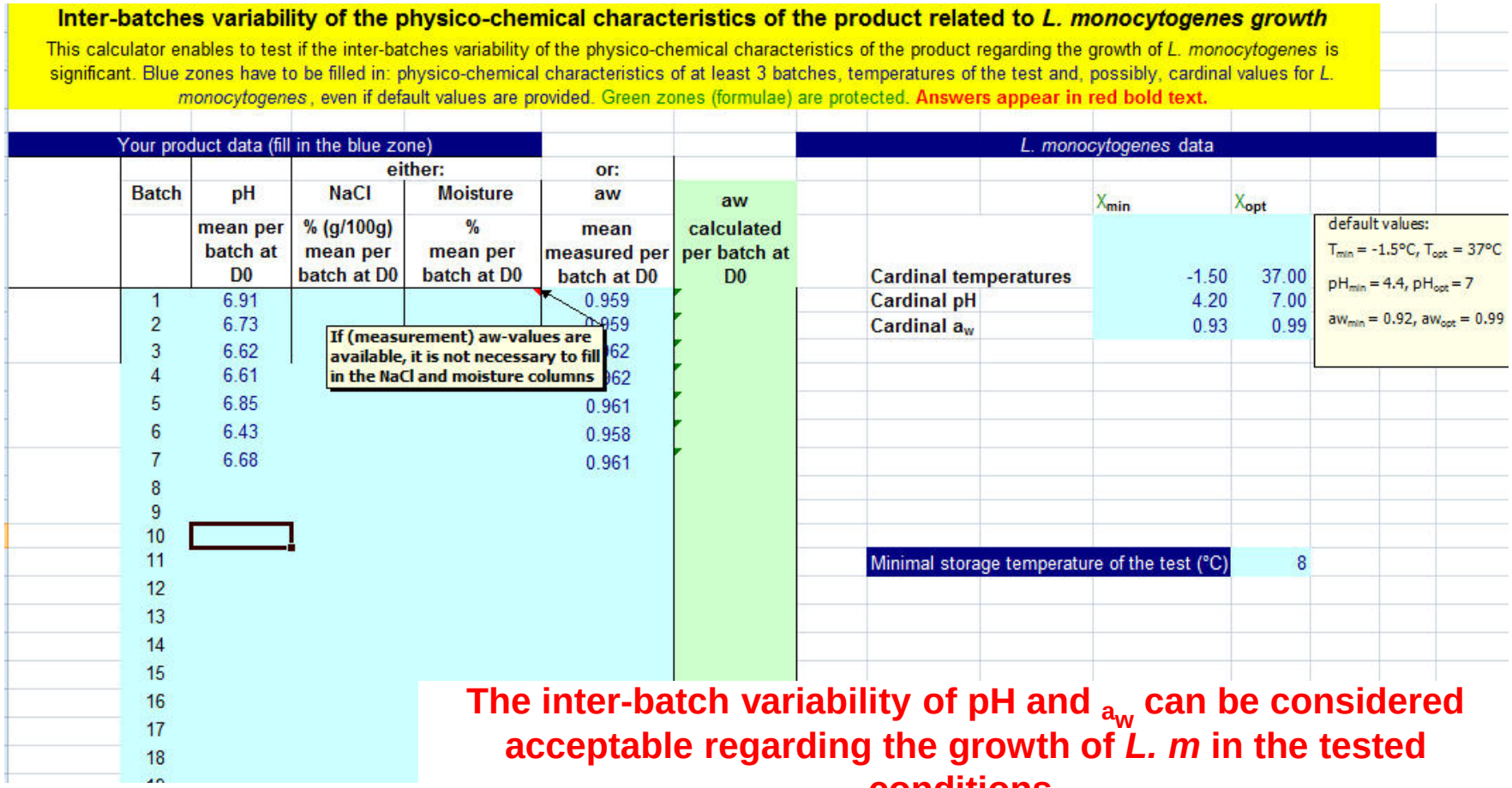
The growth probability of each of the unit tested is below 10%.



We can conclude that testing one batch may be enough

Number of batches to test

- One batch may be enough if the inter-batch variability of physico-chemical characteristics is low, regarding *Lm*.
- It is possible to get this inter-batch variability using a calculator.



Inter-batches variability of the physico-chemical characteristics of the product related to *L. monocytogenes* growth

This calculator enables to test if the inter-batches variability of the physico-chemical characteristics of the product regarding the growth of *L. monocytogenes* is significant. Blue zones have to be filled in: physico-chemical characteristics of at least 3 batches, temperatures of the test and, possibly, cardinal values for *L. monocytogenes*, even if default values are provided. Green zones (formulae) are protected. **Answers appear in red bold text.**

Your product data (fill in the blue zone)

Batch	pH	either: NaCl	either: Moisture	or: aw	aw
	mean per batch at D0	% (g/100g) mean per batch at D0	% mean per batch at D0	mean measured per batch at D0	calculated per batch at D0
1	6.91			0.959	
2	6.73			0.959	
3	6.62			0.962	
4	6.61			0.962	
5	6.85			0.961	
6	6.43			0.958	
7	6.68			0.961	
8					
9					
10					
11					
12					
13					
14					
15					
16					
17					
18					

If (measurement) aw-values are available, it is not necessary to fill in the NaCl and moisture columns

L. monocytogenes data

	X_{min}	X_{opt}
Cardinal temperatures	-1.50	37.00
Cardinal pH	4.20	7.00
Cardinal a_w	0.93	0.99

default values:
T_{min} = -1.5°C, T_{opt} = 37°C
pH_{min} = 4.4, pH_{opt} = 7
aw_{min} = 0.92, aw_{opt} = 0.99

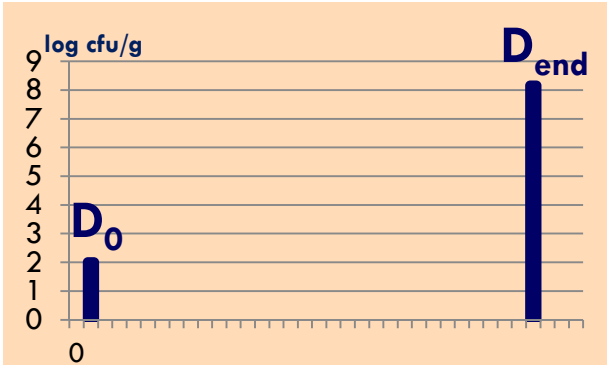
Minimal storage temperature of the test (°C)	8

The inter-batch variability of pH and a_w can be considered acceptable regarding the growth of *L. m* in the tested conditions.

Number of days of analysis

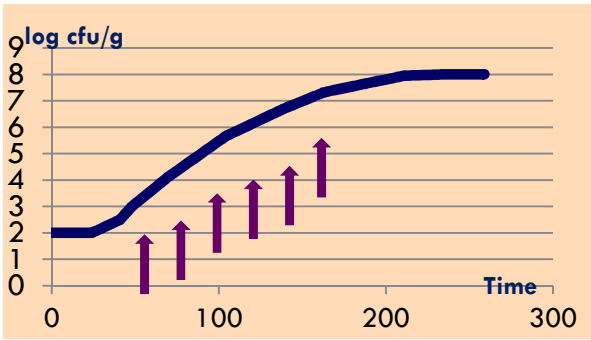
For the challenge test assessing **growth potential** (δ) :

- At least 2 days of analysis are planned:
  - one at the beginning of the test
  - one at the end of the shelf-life.



For the challenge test assessing **maximal growth rate** (μ_{max}):

- 10 to 15 days of analysis are planned:
  - at least 5/7 days during the exponential phase.



Choice of the strains

- To encompass the variability of the strains, at least 2 strains are tested:

  - One strain with known growth characteristics
    (EURL has constituted a set of strains of *Lm* from different origins characterised for their growth ability in harsh conditions)

  - Another strain: from foods, environment, outbreaks, collections.

- The strains are inoculated:

  - In mixture in case of growth potential (δ) assessment,

  - Separately in case of maximum growth rate ($Rate_{max}$) assessment.

Microbiological analyses

- The laboratory is **preferentially** EN ISO 1725 **accreditated** for the detection and enumeration of *Lm* in food.

- For *Lm* detection and enumeration, the laboratory has to use the **reference methods** for detection and enumeration of *L. monocytogenes* (Standard method EN ISO 11290-1, amended and EN ISO 11290-2, amended). It may also use **alternative analytical methods** validated or **methods certified** by a third party.

Specificity related to growth potential (δ)

Growth potential /storage

- The test units are stored under reasonable conditions of temperature:
  - using the FBO data (the 75th percentile of the observations),
  - or, temperatures fixed by DG SANCO & MSs.

Part of the cold chain	Storage temperature	Storage duration
From the manufacture to retail	8°C	1/3 of the shelf-life
Storage at retail	12°C	1/3 of the shelf-life
Storage at consumer	12°C	1/3 of the shelf-life

Growth potential /calculation

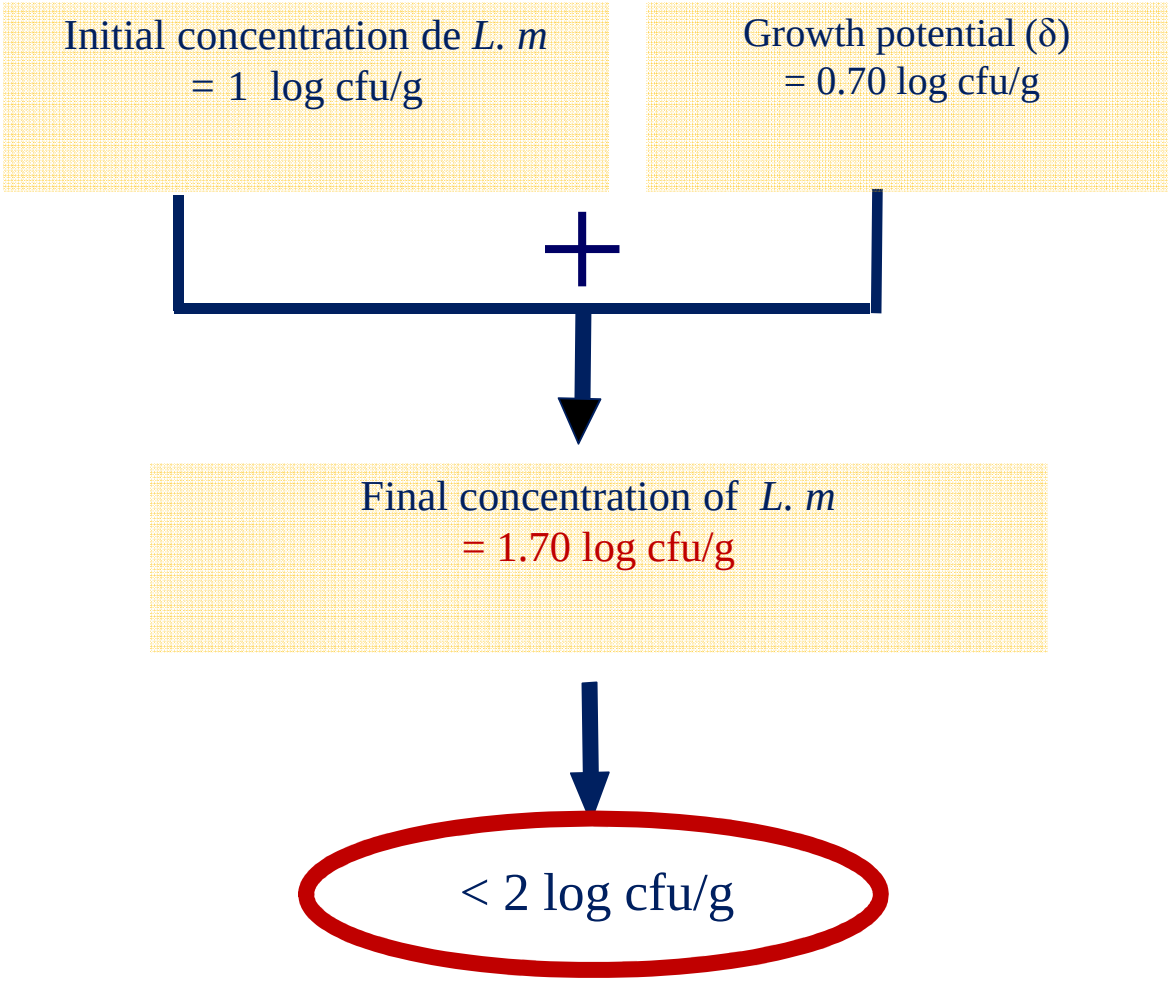
δ (per batch) = Median of the results at D_{end} – Median of the results at D_0.

Batch	Day	Concentration (log_{10} cfu/g)	Growth potentiel (δ) (log_{10} cfu/g)
1	D_0	2.08 *	0.49
		2.04	
		2.20	
	D_{final}	2.40	
		2.57 *	
		2.58	
2	D_0	2.20	0.70
		2.04	
		2.00	
	D_{final}	2.54	
		2.74	
		2.79	
3	D_0	2.08	0.40
		2.11	
		2.08	
	D_{final}	2.46	
		2.48	
		2.52	

For each batch at D0: the standard deviation between the results $\leq$0.5 log cfu/g

Growth potential/exploitation

• How to use the growth potential of *Lm* in the food (growth potential = 0. 70 $\log_{10}$ ufc/g)?



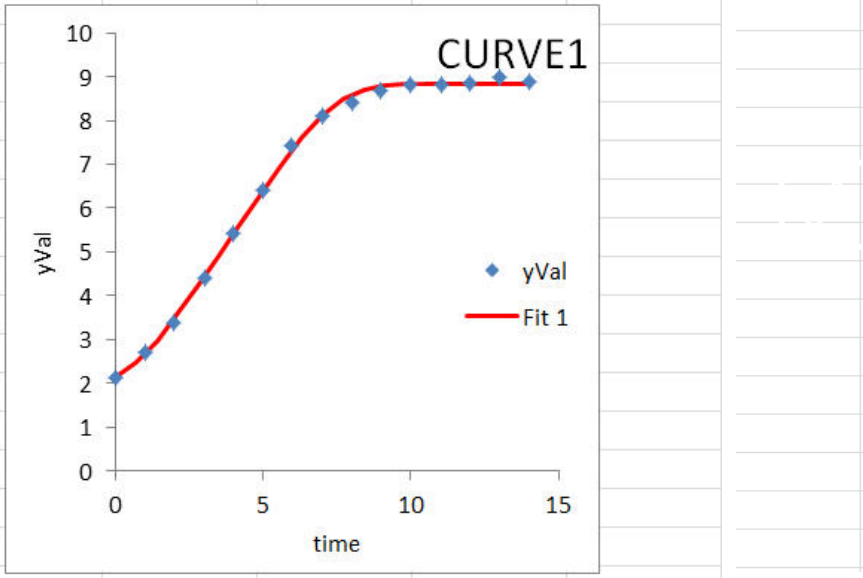
Specificity related to maximum growth rate ($Rate_{max}$)

Maximum growth rate/calculation

- Example for the calculation of $Rate_{max}$ using the ComBase fitting module.

	nCurv	rate	lag	y0	yEnd	se(rate)	l
10		1.0043873	0.7444977	2.1346342	8.8476815	0.03785	

Standard deviation



$Rate_{max}$ (log_{10} cfu/g/D) = 1.00 ± 2* 0.04 (α = 5%)

The upper limit of $Rate_{max}$ (1.08) is retained for further calculations

Durability studies

Durability study

❖ Test used to assess the growth of *Lm* in a food:

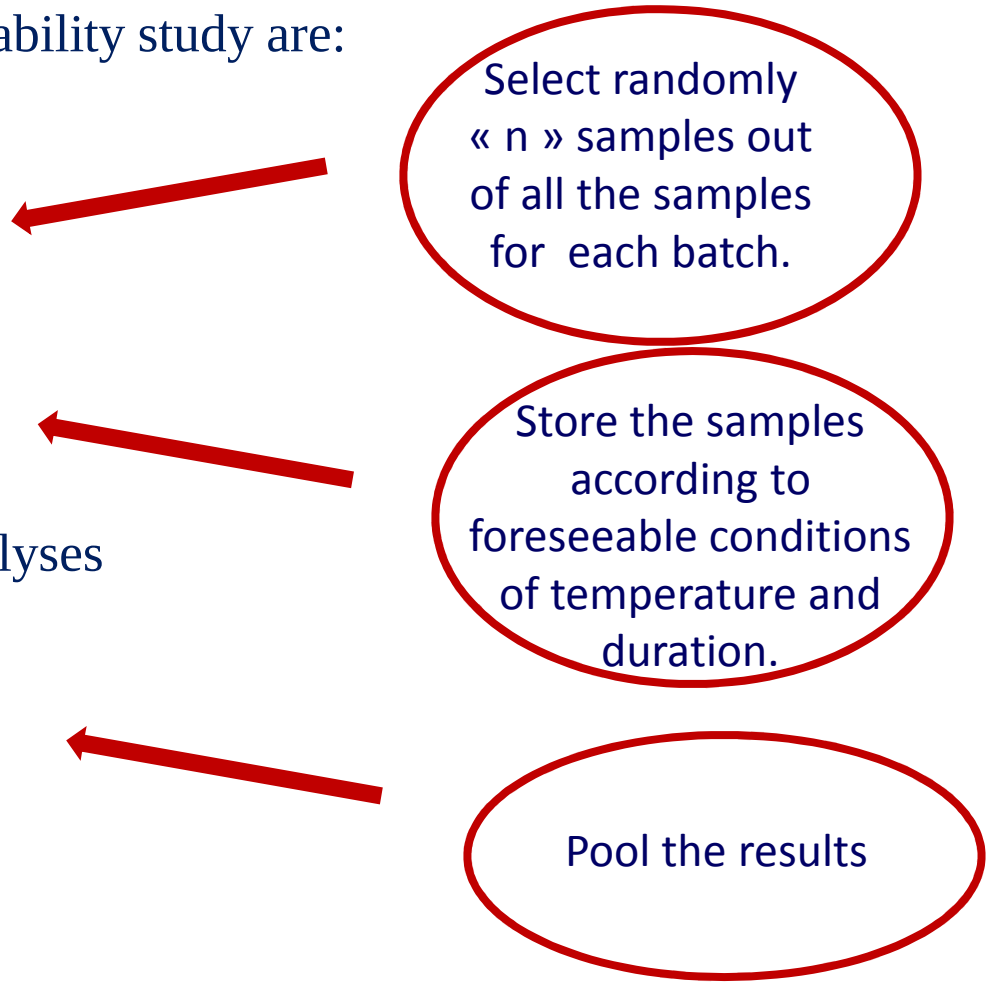
- Naturally contaminated,
- Stored at foreseeable conditions (temperature/time) from production to consumption.

Thermal profile

Durability studies

❖ The different stages of a durability study are:

- Food sampling
- Storage conditions
- Microbiological analyses
- Calculations.



Durability studies

For *Lm*, the final result is the proportion of test units over 100 cfu/g.

❖ A calculator may be used for the confidence interval

"n" Number of samples analysed	"r" Number of sam over 100 ufc	
5	0	
10	0	
15	0	
20	0	
25		
30	0	0 [0% - 11%]
35	0	0 [0% - 9.7%]
40	0	0 [0% -8.6%]
...	0	...
95	0	0 [0% - 3.8%]
100	0	0 [0% - 3.6%]
...	0	

Calculator:

Successes =

Examined =

Proportion = ////////

Confidence = 0.95

Reset Compute

Central Confidence Interval:

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

Shortest Confidence Interval:

http://www.causascientia.org/math_stat/ProportionCI.html

7. Annexes

Eight annexes

- Flow diagram describing schematically the steps from request receipt to carrying out the test, in the laboratory
- The EURL set of *Lm* strains with their growth chracteristics
- Six annexes related to the protocol :
    - Examples of preparation of the inoculum for the challenge test
    - Examples of total number of test units per batch in the frame of a challenge test to assess a growth potential
    - Some examples of contamination techniques
    - Example of impact of storage temperature on the shelf-life
    - Example of the preparation of the initial suspension
    - Examples of total number of test units per batch in the frame of a challenge test to assess a maximum growth rate, per strain, per batch

Thank you for your attention!