Pharmaceutical industry

Food processing industry

Cosmetic industry

GBA

Methods of microbiological analysis

An overview and selection criteria

Cours
14/09 (8 - 9h30)
Cours en ligne
24/09 (11h30 – 13h)
1/10 (15h15 - 16h45)

	TP1	TP2	TP3
groupe1	28 et 30/09	2, 4 et 6/11	23, 25 et 27/11
groupe2	21 et 23/09	12, 14 et 16/10	16, 18 et 20/11
groupe3	30/09 et 2/10	19, 21 et 23/10	9, 12 et 13/11

Premier contrôle écrit sans doc 1/12/20

Microbiological analysis (1/3)

a valuable tool

Applies to bio-catalysts, pathogens, indicator microorganisms[1] and spoilage microorganisms

Involves the use of biological, biochemical, molecular or chemical methods for the detection, identification or enumeration of microorganisms.
They are either reference[2] or alternative[3] methods

Why is microbiological analysis important?

- To check the raw material/product quality, the environment (process equipment, ambient air, handlers, water) and the end-products
- To monitor and control manufacturing strategy with corrective actions
- To monitor and control the safety of products placed on the market
- To optimize production
- To limit product recalls (slide 9)

What is behind the development of alternative methods

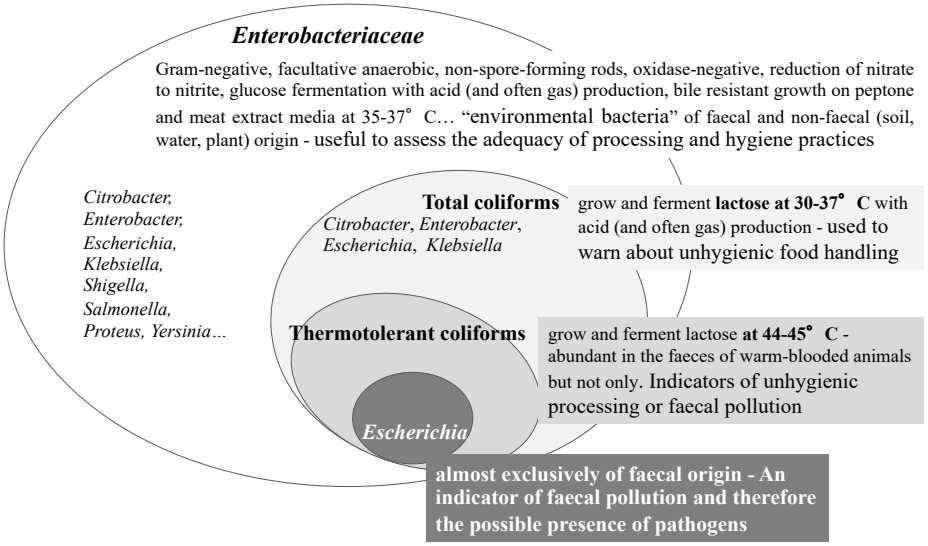
- The repeated health crises and product recalls in the food sector
- The imperatives set by statutory texts (regulation EC)
- Consumer requirements for the quality and safety of products
- The need to produce more, to release products as quickly as possible…

[1]Or bioindicators
[2]Standardized methods (e.g. ISO methods) the reference analytical methods for official control
[3]New methods at least equivalent to the reference method (proprietary or non-commercial)

ISO International Organization for Standardization

Examples of bioindicateurs

Enterobacteriaceae, coliforms and *Escherichia coli*



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Microbiological analysis (2/3)

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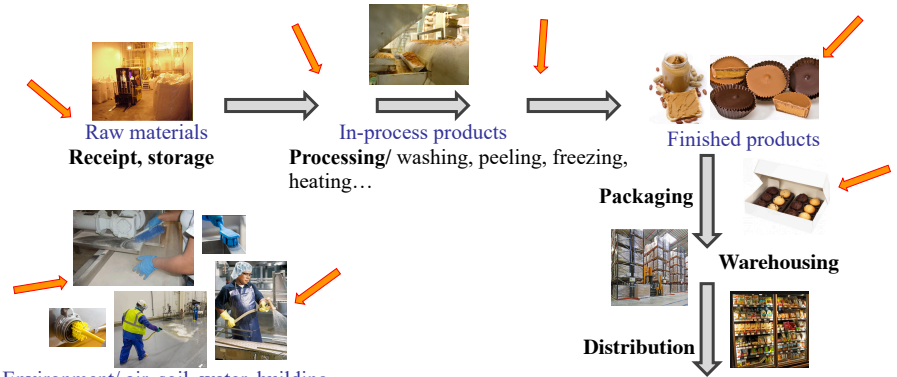
ISO International Organization for Standardization

4

Microbiological analysis

A valuable tool

Process flow diagram and areas monitored in a standard microbiological surveillance program



(1) The removal of soil from a surface or equipment to give it a visibly clean appearance
(2) The reduction of microorganisms on a cleaned surface to a level that is generally regarded as safe

Microbiological analysis (3/3)

a valuable tool

Applies to bio-catalysts, pathogens, indicator microorganisms(1) and spoilage microorganisms

Involves the use of biological, biochemical, molecular or chemical methods for the detection, identification or enumeration of microorganisms.
They are either reference(2) or alternative(3) methods

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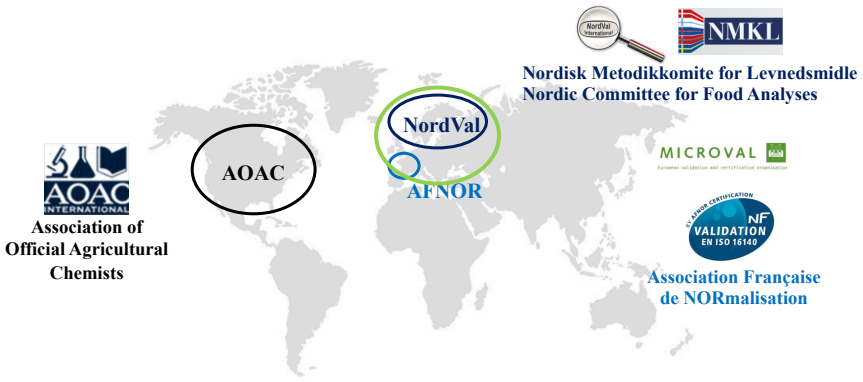
International Organization for Standardization

International certification organisations for the validation of alternative methods



- To show that a proprietary method performs equally well as the (internationally standardised) reference method
- To ensure that health, safety and environmental requirements are met…

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Microbiological analysis methods

At present, various methods and equipment more or less sophisticated are offered **but which do you choose?**

The **Objective of this course** is to give you an overall idea, through the use of a number of examples, of different techniques available to choose a method adapted to the given production

Main skills targeted…

- be able to choose a method according to a given production
- be able to analyse the results obtained in order to implement corrective actions

… by keeping in mind

- No universal or ideal microbiological method of enumeration and identification, but several available methods
- Each method has its strengths and weaknesses

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Regulation EC 2073/2005
November 15, 2005

Regulation EU 2019/229
February 7, 2019

Microbiological criteria

Definitions and application stages

Microbiological criterion defines "the acceptability of a product, a batch of foodstuff or a process, based on the absence, presence or number of microorganisms, and/or on the quantity of their toxins/metabolites, per unit of mass, volume, area or batch"

Microbiological criteria

Process hygiene criteria

"indicating the acceptable functioning of the production process. An **indicative contamination value** above which corrective actions are required in order to maintain the hygiene of the process in compliance with food law"

- Applied at the end of the manufactured process
- Can be applied to several steps of the production process

Food safety criteria

"defining the acceptability of a product or a batch of foodstuff, applicable to products placed **on the market**"

- Available during the entire shelf-life of products

http://nf-validation.afnor.org/wp-content/uploads/2014/04/Reg_CE_2073-2005_fr.pdf
http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:2005R2073:20071227:EN:PDF
http://agriculture.gouv.fr/denrees-alimentaires-criteres-microbiologiques-dhygiene-des-procedes

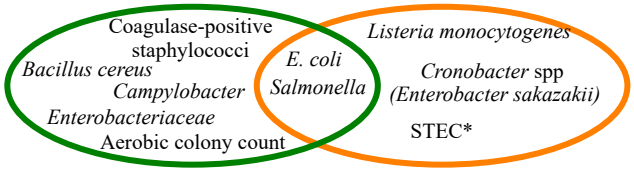
Regulation EC 2073/2005
November 15, 2005

Regulation EU 2019/229
February 7, 2019

Microbiological criteria

Microorganisms

"Microorganisms" means bacteria, viruses, yeasts, moulds, algae, parasitic protozoa, microscopic parasitic helminths, **and their toxins and metabolites**



Process hygiene criteria = bioindicators

The effectiveness of raw material quality, GMP (good management practices) and sanitation programs

No listed:
- Lactic acid bacteria
- Coliforms
- Yeasts/Molds
- *Pseudomonas*
- Micrococci
- ...

Food safety criteria = pathogens

Presence/**Not detected** testing. Zero tolerance in finished products

No listed:
- *Clostridium perfringens*
- *Vibrio* spp
- *Yersinia enterolitica*
- Hepatitis A virus, Norovirus
- ...

http://faolex.fao.org/docs/pdf/eur61603.pdf
https://www.producteurs-fermiers-isere.fr/IMG/pdf/ce_2017_1495_campylobacter.pdf
https://www.fsai.ie/uploadedFiles/Reg1441_2007(1).pdf
https://eur-lex.europa.eu/eli/reg/2019/229/oj

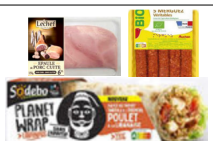
*Shiga Toxin-producing *E. coli*

Examples of product recalls

in recent months

A **food recall,** action taken by a business to remove unsafe food from distribution, sale and consumers following an inspection *e.g.* by the DGCCRF*

Raw milk and dairy products



Meat and products thereof

Frozen vegetables, fruits and products thereof

Eggs

Baking products

Cosmetic products

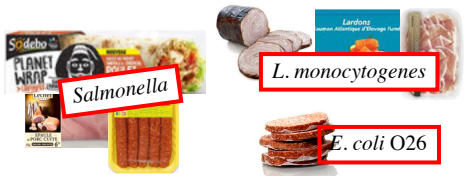
http://alimentation.gouv.fr/alerte-produit1
http://www.economie.gouv.fr/dgccrf/securite/alertes/Rappels-de-produits
http://www.quechoisir.org/rubriques/rub-produits-au-rappel
http://www.60millions-mag.com/rappel
https://www.economie.gouv.fr/cedef/retrait-rappel-produits-dangereux

*Direction Générale de la Concurrence, de la Consommation et de la Répression des Fraudes

Examples of product recalls

Microbial contamination

Raw milk and dairy products



Meat and products thereof

Eggs

Baking products

Frozen vegetables, fruits and products thereof

Cosmetic products

*Aerobic Colony Count

Examples of product recalls

Microbial contamination

Raw milk and dairy products

Meat and products thereof

Eggs

Baking products

Gas-producing thermophilic bacteria

Frozen vegetables, fruits and products thereof

Microbial contamination

Cosmetic products

*Aerobic Colony Count

Microbial growth

Factors or some tracks

Intrinsic factors: Moisture content, pH and acidity, nutrient content, biological structure, redox potential, naturally occurring and added antimicrobials, competitive microflora, growth…

Extrinsic factors: types of packaging/atmospheres, time/temperature, storage/holding, processing steps…

	aw	T° C	pH
Salmonella spp growth	> 0.94	5 – 45/47	4.2 - 9.6
L. monocytogenes growth	> 0.92	0 – 45/50	4.4 - 9.4
S. aureus growth	> 0.83	7 - 48	4 - 10
S. aureus toxin	> 0.88	10 - 48	4.5 - 9.6

Limits for growth when other conditions are near optimum

Fish
aw > 0.99
pH 6.6 – 6.8

Dry sausage
aw 0.85 – 0.95
pH 5.0 – 5.5

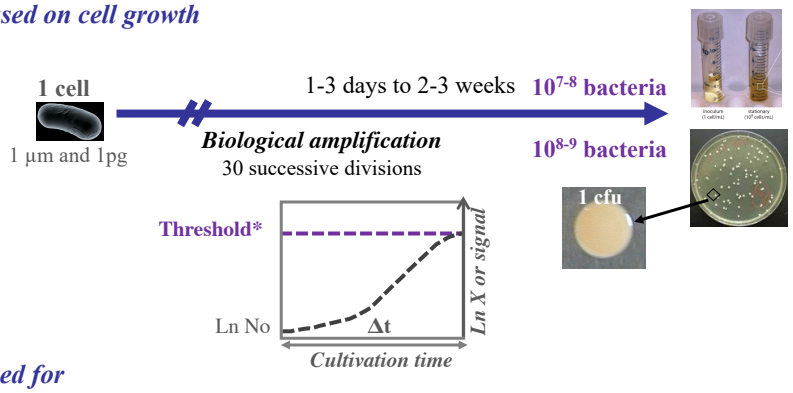
Fruits/apple
aw 0.97
pH 2.9 - 3.3

Eggs
aw 0.97
pH 6.0 (-> 6.9)

Vegetables/onions
aw > 0.97
pH 5.3 – 5.8

Conventional microbiological methods (1/3)

Based on cell growth



Used for

- Quantitative analysis: Enumeration (indicator microorganisms) in relation to a set limit (legal restriction) or not (growth monitoring)
- Qualitative analysis: Research and identification, the question is whether an organism is present in a given matrix or not (presence/absence testing)

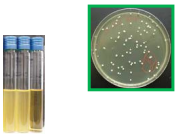
*the value from which the response is detected

Conventional microbiological methods (2/3)

Analysis time

Quantitative analysis

Sample — Preparation (0.5-1 h) — *(Revivification) (1-3 h)* — Detection/ enumeration **24-72 h***



Data at least 1-3 days

Qualitative analysis

Sample — Preparation (0.5-1 h) — Selection: *Pre-enrichment 16-24 h** / *Enrichment **24-48 h**** — Detection **24-48** h* — Identification: biochemical characteristics and serology **24 h*** ++

Data at least 3-5 days

* Detection time= f (microorganism, detector)

➡ **(1) The critical and limiting stage is the response time due to cultivation time**

Conventional microbiological methods (3/3)

Other characteristics

- **Specificity**, the ability to detect the target microorganism without interference with matrix components or others microorganisms
- **Accuracy**, the degree to which the result of an experiment agrees with the « true » or expected result

- Qualified personnel requirement, labor-intensive, time consuming
- No detection of cells in the Viable But Non-Culturable (VBNC) state

⇒ **(2) These methods are reference methods to validate new methods and to control quality**

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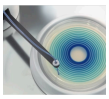
Improvement of conventional methods (1/3)



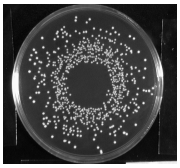
- **Dilution/Inoculation/Enumeration** with an automatic diluter and plater

1) Exponential mode: The liquid sample is dispensed according to an Archimedean spiral with a logarithmically decreasing volume

Volume of sample at every point of plate is known

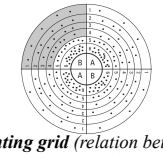
A **fixed** sample volume plated onto the Petri dish

Incubation →



At least **4 dilutions** on a single Petri dish (from 30 to $4*10^5$ CFU/mL)

Manual counting →



Counting grid *(relation between CFU and volume)*

Automatic counting →

Automated colony counter

- ✓ Significant reduction in cost per test, practice time and use of consumables
- ✓ Repeatable and reproducible results up to 99 %
- ✓ 25 seconds for a full cycle (disinfection, sample-taking and plating)
- ✓ A fully validated Microbiological method (**ISO 7218**/AOAC 977.27/ FDA-BAM Ch.3)

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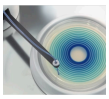
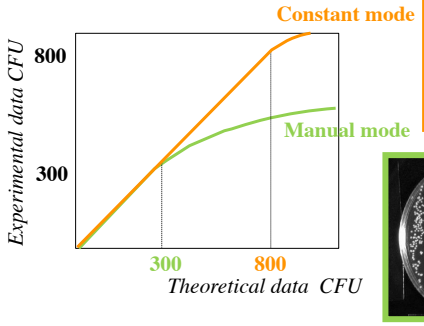
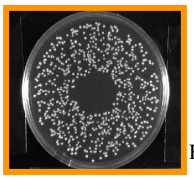
Improvement of conventional methods (2/3)



➢ **Dilution/Inoculation/Enumeration** with an automatic spiral plater

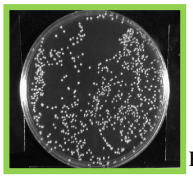
2) Constant mode: The stylus arm dispenses liquid sample uniformly across the plate





For more precision

Homogeneous repartition



Heterogeneous repartition

Applications: food, agro-food, cosmetic, veterinary, medical, environmental, chemical industries and public research institutes…

Various devices: Spiral plater (*Wasp, AES laboratory*), EasySpiral (Interscience)…

Improvement of conventional methods

Selective-differential media

Selective media support the growth of some microorganisms while inhibiting the growth of others (SDS, bile salt, antibiotics, dyes…)

Differential media are used to visually distinguish microorganisms from one another based on growth characteristics (sugar fermentation by acid-base indicators…)

Composition of two selective-differential media for coliforms (grams per liter)

		VRBL
Substrates	Peptone + YE	+
Selective agents	Bile salts	1.5
	Crystal violet	0.002
Differential agents	**Lactose**	+
	Neutral red (pH indicator)	+
Gelling agent	Bacteriological agar	+

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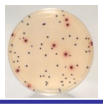

Composition of two selective-differential media for coliforms (grams per liter)

		VRBL	Chromogenic medium
Substrates	Peptone + YE	+	+
	Growth promoters + Buffer system	-	+
Selective agents	Bile salts	1.5	0.8
	Crystal violet	0.002	-
Differential agents	**Chromogenic mix**	-	+
	Lactose	+	-
	Neutral red	+	-
Gelling agent	Bacteriological agar	+	+

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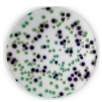
The difference is clear

Selective-differential medium

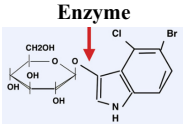
based on hydrolysis of chromogenic substrates



Chromogenic substrates
Soluble colourless substrate

Transport across cell membranes

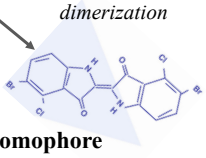
Enzyme



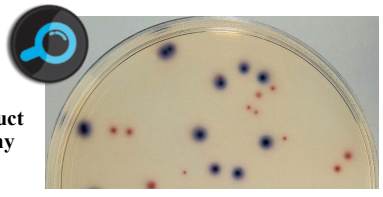
X-β-D-glucoside

5-bromo-4-chloro-3-indolyl-β-D-glucoside

Oxidative dimerization



Coloured product located at colony



Chromophore
Insoluble coloured product

Simultaneous enumeration or detection of 2 target microbial groups or types in agar media
e.g. E. coli and coliforms

http://www.chromagar.com/p-chromogenic_agar_technology.html#.XxrU7S3pOu4

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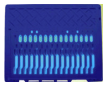
Selective-differential medium

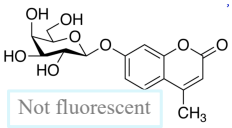
based on hydrolysis of fluorogenic substrates



For bacterial growth quantification **in broth media**
Enumeration by the MPN method

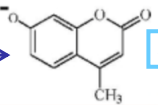
7-hydroxy-4-methylcoumarin

Total viable count
E. coli



Not fluorescent

Bacterial specific activity

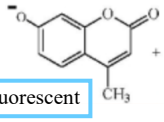


Fluorescent

4 MU-X or MUG
(4 methylumbelliferyl ß-D galactopyranoside)

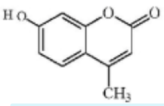
+ X or galactose

Enteriobacteriaceae
coliforms



fluorescent

Media acidification due to carbohydrate metabolism



Not fluorescent

Various suppliers: Petrifilm-*3M*, Compact Dry-*HyServe*… SimPlate-*BioControl,* RAPID'*E.coli* 2 or RAPID'*Salmonella*,-*Bio-Rad,* Tempo, *Biomerieux*, Colilert, *Idexx*…

Improvement of conventional methods (3/3)

Once the samples are prepared, many different automation options or ready-to-use media are available

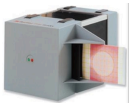
- Automatic dispensers
- Automated plate pouring system

Compact Dry

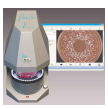
- Ready-to-use media

(dehydrated media or in ready-to-use formats)

Petrifilm



- Facilitated analysis of results (automated dilutors and counting devices, image analysis, laser counter…)

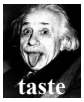


Reduced workload and ease of operation
Reduced time requirement to perform test and obtain results (18-24 h in the best case)
But the critical and limiting stage is the response time due to cultivation time

How to improve the microbiological analysis ?

⇨ **By reducing the response time (Δt)** ***i.e.*** **by decreasing the threshold**
i.e. **by increasing No**

Human detectors



Chemical signals | Optical signals

1 cell

Δt

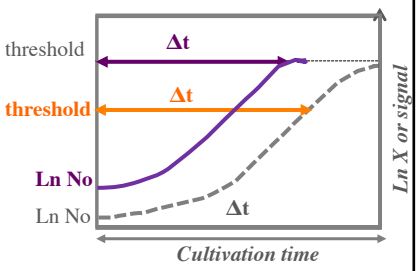
10^{5-7} 10^{7-8}

10^{8-9} CFU
Threshold

1. Detect a stronger signal than a cell with more sensitive detectors

- from target metabolic markers by physico-chemical and biochemical methods
- from target cellular elements by immunological and genomic methods

2. Detect a stronger signal than a cell with a higher cell initial charge



New methods

and many devices

These advances in technology make detection and identification faster

More convenient

Which one to choose?

More specific

More sensitive…

Than conventional methods

⇨ **Based on the answers to several preliminary questions as what have you been doing so far? and how would you like to improve it?**

Some preliminary questions

1. **What do you need to test for (pathogens, indicators, spoilage organisms, biocalysts)?** fully manual or automation
   Target
2. **How many tests will you do per day?** number of samples per run, per hour **Frequency**
3. **How fast do you want results?** time taken to prepare and conduct the test, time to result
   Time
4. **What skill level is required for a new test?** a "black box" type test or data with a personal interpretation
   Control or research
5. **What are you willing to spend on specialist equipment in order to do a new test?** How much budget do you have? Operating and investment costs of the rapid method…
6. **What consumable cost are you (and your clients) willing to accept?** Expensive consumables drive up the cost per test
   Cost
7. **What are the servicing/calibration costs for the new method?**
8. **Will the method work? Which technologies are 'mature'?** who else is using them? Company reputation and technical service. Simplicity of use
9. **Is the method recognized by the regulation?** Acceptability of the method validated and certified to ISO standard
   Recognized, validated or not

…

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