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Course handout:

MICROBIOLOGICAL CONTROL TECHNIQUES

3rd Year Microbiology

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Foreword

This manuscript is intended for students of the third year of natural and life sciences, specializing in Microbiology. Its objective is to provide students with the essential theoretical and practical knowledge in Microbiology, but also to master microbiological quality control according to the criteria of the Algerian standard (failing that, the international standard). It is designed for a fairly simple and easy reading.

It begins with an introduction, then it deals with the notion of quality and its two components (hygienic and technological), as well as the control policy. Afterwards, it describes the collection, transport and preparation of samples, once completed, a section is devoted to classical counting techniques (microscopic, in solid media and in liquid media), but also to rapid detection techniques, which requires having a later section dealing with the phenetic identification of microorganisms by gathering cultural, morphological, biochemical, physiological, immunological and molecular characters. After that another section reveals the control of raw materials, sourdoughs, manufacturing, cleaning and disinfection and finished products.

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List of abbreviations

AFNOR:AssocFrench standardization initiative
BCPL: Bromocresol purple lactose broth
CMI: Minimum Inhibitory Concentration
H2 O2: Hydrogen peroxide
HCO3: Hydrogen carbonate
ISO: International Organization for Standardization
KNO3: Potassium nitrate
KOH: Potassium Hydroxide
LDC: Lysine decarboxylase
N / A: Algerian standard
NaCl: Sodium chloride
NH2: Amide ion (amine)
NPP: Most probable number
NR: Nitrate Reductase
O2: Dioxygen
OGA: Oxytetracycline Agar Agar
ONPG: Ortho Nitro Phenyl Galactopyranoside
TDA: Tryptophan deaminase
TSE: Tryptone salt water
RM: Methyl Red
VP: Voges Proskauer
VF: Meat liver agar

UNITS:
g Gram
mL Milliliter
mm Millimeter
mm^3 Cubic millimeter
cm2 Ccubic wholemeter
min Minute
s Second
gL-1: Gram per liter
M: Molar
rpm: Rounds per minute
UFC/gr: Colony Forming Unit per gram

1. General

1.1 Origin and nature of the microbial flora of foods

The presence of microorganisms in foods that have not undergone antimicrobial treatment is completely normal. With some exceptions (some products such as the inside of an egg are naturally sterile), raw food material contains microorganisms, and the microbial load can be relatively high, in the order of 102 to 106/g. Raw food material is of plant or animal origin and the flora associated with it is therefore that naturally present on plants and animals respectively. Furthermore, many exogenous inputs can increase the microbial load. The original flora is mostly made up of saprophytic commensal microorganisms; however, germs that are actually or potentially pathogenic can be found there (sick plants or animals or "healthy carriers"). Food is exposed to different sources of microbial contamination. For example, plants are contaminated by air, soil, water, fertilizers, etc. Technological handling and treatments are also involved. Handlers are responsible for contact contamination or indirect contamination. While the presence of an original flora can be difficult to avoid, that linked to contamination can be greatly reduced by hygiene. During manufacturing operations, the flora evolves qualitatively and quantitatively. Certain treatments are carried out with the aim of inhibiting or destroying the flora in whole or in part (antimicrobial treatments).

1.1.1 Flora from animals and plants

We can distinguish between normal flora, found in healthy subjects (commensal flora) and pathogenic flora found in sick subjects. The commensal flora of animals and plants are of significantly different types. However, the surface flora can present similarities because it comes from environmental contaminants: air, water, soil, etc. The pathogenic flora is totally different: the plant flora has a metabolism rather oriented towards carbohydrates, while that of animals is towards proteins. Plants have a microbial flora rich in yeasts {Saccharomyces, Rhodotorula, Candida, etc.) and molds {Saprolegnia, Plasmodiophora, Mucor, Rhizopus, Fusarium, Aspergillus, Penicillium, etc.). The bacteria they contain mainly belong to the group of Gram-bacilli (Pseudomonas, Xanthomonas, Flavobacterium, Acetobacter, Enterobacter, Erwinia, etc.) and to that of Gram-positive non-spore-forming bacilli (Corynebacterium, Lactobacillus, Leuconostoc, Streptococcus, Pediococcus, etc.). Animals have different types of commensal flora. The most important are the surface flora (micrococci, corynebacteria, Listeria, aerobic spore-forming bacteria, etc.) and the intestinal flora (colilorms, enterococci, anaerobic spore-forming bacteria, but also lactic acid bacteria such as

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Bifidobacterium, Lactobacillus, etc.). The intestine of humans or animals contains up to 1011 germs/g. The respiratory and genital tracts and the udder also contain abundant flora (lactic acid bacteria). Phytopathogenic flora are often fungal, but some bacterial genera play an important role (Pseudomonas, Erwinia, Corynebacterium, etc.). The pathogenic flora of animals is mainly composed of bacteria (Mycobacterium, Brucella, Listeria, Staphylococcus aureus, some Streptococcus and pathogenic Enterobacteria such as Salmonella, Shigella, Yersinia, etc.). There are significant differences between the different types of plants and animals. Some microbial species are typical of a particular host, both for saprophytic and pathogenic germs. The flora also varies according to age (or stage of development), nutritional conditions, environment, treatments (medicinal for animals, phytosanitary for plants).

1.1.2 Contamination by handlers

The commensal and pathogenic flora of humans are similar to those of animals. Contamination can come from healthy people as well as sick or cured people (healthy carriers). The skin in general, hair and other body hair are very rich in microorganisms (10 2 to 10 4 germs/cm2 for the skin). Contamination by handling is first of all contact contamination, mainly on the hands. The germs incriminated are mainly Staphylococcus, Streptococcus, Galkya, etc. which are carried by healthy skin or by wounds, abscesses or boils. Lack of hygiene can lead to the presence of intestinal bacteria on the skin (fecal contamination: Salmonella). Aerosol contamination (coughing, sneezing, but also simply breathing) can also occur: germs of angina, sinusitis, both bacterial (streptococci, staphylococci, etc.) and viral. In addition, contamination can be linked to clothing.

1.1.3 Environmental contaminants

Air and especially soil are rich in micro-organisms. Air contains dust loaded with fungal spores or conidia, bacterial spores (Bacillus) and non-spore-forming bacterial forms (micrococci). Soil and in particular topsoil contain a very large number of microbial species of very diverse types (Bacillus, Clostridium, Streptomyces, Corynebacterium, spores and conidia of Penicillium, Aspergillus, Mucor, Fusarium, etc.). Fresh water and salt water contain a variable number of micro-organisms depending on the intensity of the pollution. Their natural flora consists of aerobic Gram bacteria - including Pseudomonas, Vibrio, Aeromonas, Zooglea, etc. Water is used extensively in the food industry: this water can contain various

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micro-organisms and be the source of contamination. Microorganisms found in water in addition to the normal water flora can have various origins: soil (Streptomyces, Bacillus, etc.), fecal matter (Enterobacteria, streptococci, etc.), plants (fungal spores and conidia), animals, etc. Water can be the vector of pathogenic microorganisms: Salmonella, Shigella, Yersinia, Vibrio, Listeria, viruses, protozoa, etc. The contribution of microorganisms from the environment can be done directly or via vectors (insects). Wastewater is a veritable breeding ground for microbial cultures with flora of various origins and generally a high rate of fecal germs.

1.1.4 Industrial contaminants

Industrial equipment is a source of contamination, particularly porous surfaces (worktops), tools and machines, fabrics (tea towels, various canvases), etc., as well as floors and walls. Industrial contamination is generally specific to a given industry. Cleaning habits and the nature of the products used are sometimes very important: they can allow the selection of a given contaminant. When preparing products from various raw materials, some of these constitute a privileged supply of micro-organisms: some find in the mixture produced favorable conditions that they did not encounter before (for example contamination by spices, contamination of an animal product by a plant product, etc.). Treatments can induce or promote the dispersion of flora: for example, grinding a seed to make flour will bring the surface flora into contact with the interior: mincing meat or mechanically tenderizing it can have the same result. In butchery, slaughtering and extraction cause significant bacteremia if they are not carried out satisfactorily. The manufacturing conditions will also "select" various categories of microorganisms: thermophiles, thermoresistant, psychrophiles, acidophiles, etc. The storage and preservation conditions influence the physicochemical conditions and the possibility of new contaminations.

Industrial waste is also a particularly significant potential source of contamination. Care must be taken to properly separate the different manufacturing phases and isolate this waste.

1.2 Evolution of the flora

1.2.1 Factors of evolution

The behavior of the microbial flora will depend on several types of factors:

- the level of initial contamination: the higher it is, the earlier and more significant the activity will be (the latency time is shortened);

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- the properties and requirements of the micro-organism: ability to degrade substrates (which depends on the species incriminated, but also on the enzymatic equipment and the orientation of the metabolism; there may be adaptation phenomena), nutritional requirements (some micro-organisms have low requirements, prototrophy or complexes, auxotrophy), development conditions, resistance or sensitivity to various factors or products, ability to compete with other flora, etc. Some micro-organisms can only develop on certain categories of product, others have a much broader "development spectrum";

A prototrophis a living organism capable of proliferating in a basic environment without requiring the presence of particular growth factors; it itself synthesizes the substances necessary for its proliferation.

- the nature of the food: structure (presence of teguments or protective structures, internal texture, viscosity, diffusivity in relation to gases, products, etc.), water content (water activity, pressure), nutrient composition (character "poor", "rich", balanced, nature of the main source of carbon and nitrogen, presence of vitamins and growth factors), presence of natural or artificial inhibitors (many products contain natural inhibitors, tannins, polyphenols, organic acids, essential oils, etc.), pH (this parameter is a fundamental factor: a low pH is generally unfavourable to pathogenic micro-organisms, etc. Some foods allow easy development for many categories of micro-organisms, while others are hostile environments allowing the development only of specialised flora;

- environmental conditions: nature of the atmosphere, humidity, temperature, etc.;

- technological treatments: these treatments will often modify the texture, pH, water content and sometimes the composition of the food; moreover, they can modify the environmental conditions.

1.2. Activity of microorganisms

The development of a microorganism will affect the intrinsic quality of the food and therefore its commercial value. The changes are not always harmful, and when they are, they are not necessarily dangerous for the health of the consumer.

The number of microorganisms in a food cannot necessarily be considered as an indicator of poor health quality. Abundant development sometimes has interesting consequences. Some microorganisms are useful and even essential: they participate in the development or transformation of the food, ensure the development of particular organoleptic

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qualities or participate in preservation and promote hygienic quality by preventing the development of dangerous germs. Other germs are harmful to the quality of the food itself at the level of manufacture or preservation.

These are the common contamination germs that can cause serious problems in the industry. Useful germs, mainly yeasts and lactic bacteria, but also acetic and propionic bacteria and certain molds, are very rarely involved in health accidents. Common germs, not participating in useful fermentations, can have various harmful effects that affect the food and commercial value of products (modification of texture and appearance, alteration of food value, alteration of organoleptic qualities, degradation of packaging, etc.). These germs can also, under certain conditions, prove dangerous for health by being responsible for poisoning due to the formation of toxic substances (amines), or even benign intestinal infections or toxi-infections: these strictly pathogenic germs are dangerous even in small quantities and in the absence of development or degradation induced in the food.

2. Objective of microbiological control

The microbiological control of food or any other consumable product aims to control the less apparent but fundamental characteristics of a consumable product.

This concerns wholesomeness, i.e. the absence of toxic action, pathogenic or toxigenic microorganisms as well as the level of populations of spoilage germs. Furthermore, in the case of preserves, it controls the stability of the products, i.e. the ability of the product not to deteriorate too quickly if the storage conditions are respected.

Consequently, the control aims to detect batches of food products whose level of contamination flora populations exceeds the threshold tolerated by the standards in force. Given that biological sterility is impossible at the risk of altering the nutritional values of the product, the quantitative factor intervenes at the level of the analysis of this flora. Most often, qualitative knowledge of the composition of the flora is useless except in the manufacturing circuits to detect the agent responsible for the manufacturing accident.

The presence of pathogenic germs is totally undesirable in food products because of the health risk that this type of product poses for the consumer. In this case, it is the qualitative analysis that takes precedence. And the detection of a single pathogenic germ makes the product unfit for consumption (safety analysis).

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Therefore, the control aims to assess the quality of the products.

2.1. Definition of quality

Quality is what characterizes (qualifies) an object, a living being, without any moral precision, without any positive or negative assessment… The notion of quality includes many facets, as may be required for the description of elements as diverse as wine, diamonds, behavior, beauty or ugliness, etc. (Insert "The different facets of quality"). And yet, since the 16th century already, and with the exception of philosophical and logical uses, the word quality has taken on positive connotations, as in the recent expressions "quality of life" or "value for money". This extension of the meaning, almost an abuse of language, is widely used in current everyday language, and particularly in the advertising field.

Between several partners. Implicit or explicit, it will serve as a basis for establishing a standard, a value, a price, determined from characteristics defined in the transaction. As a result, there is not one but multiple qualities, not necessarily defined as positive. The notion of quality is not absolute, it is relative and changing, closely linked to industrial developments, economic movements and, more broadly, to the history of companies.

In the case of food, quality can concern multiple, extremely different elements: origin, composition, manufacturing process, rarity, sensory aspects, safety, regulatory framework, etc. This list is, of course, far from exhaustive, and each of the elements is characteristic of a quality as soon as it is defined.

2.2. Components of quality

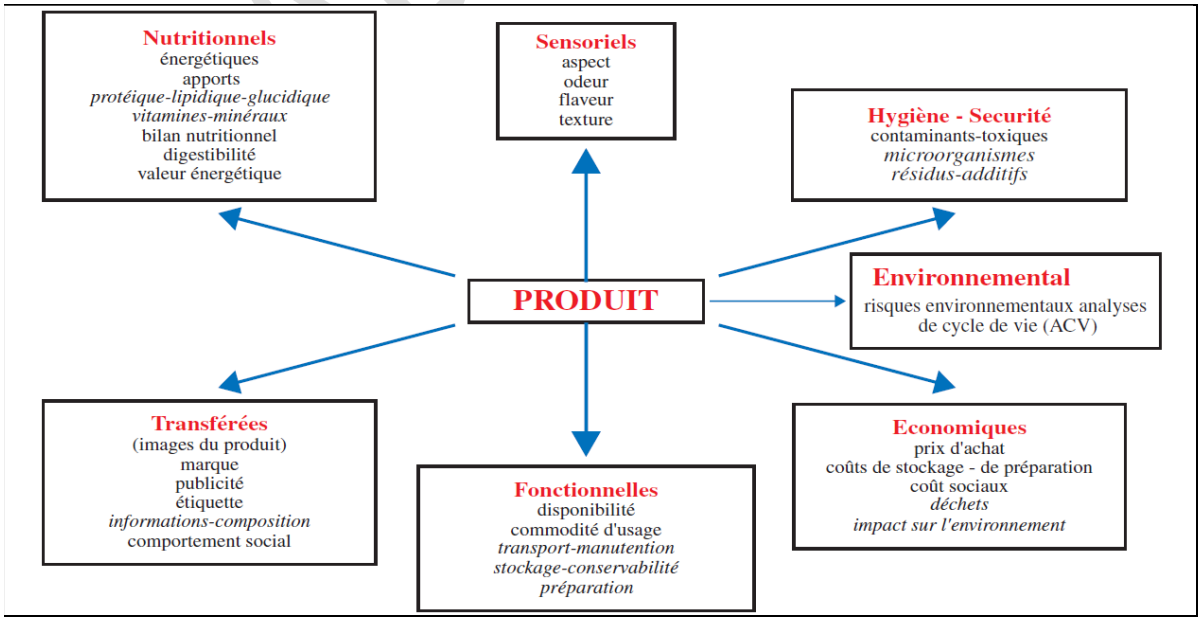

Figure 01: The components of quality

2.2.1. Hygienic Quality

The hygienic quality of a food product is the absence of pathogenic microorganisms or their toxins that could harm the health of the consumer. The presence of such microorganisms and their toxic compounds leads to food-borne diseases. Depending on the nature of the microorganisms involved, three cases of disease may occur:

Food infections: set of symptoms after ingestion of a quantity of spoiling microorganisms living in the food product or in water. This is the case for example of Enteropathogens or viruses: Salmonella enterica (salmonellosis*), Shigella spp. (bacillary dysentery), Yersinia enterocolitica (yersiniosis), enteropathogeni*c E. coli, and viral infection.

Food poisoning: set of symptoms after ingestionof a quantity of pathogenic microorganisms living in the food product and the secretion after ingestion of a toxin. This is the case for example of: Clostridium perfringens and Bacillus cereus (gastroenteritis) and *Vibrio cholerae* (cholera).

The latter two are manifested by diarrhea, vomiting, abdominal pain and are associated with fever and disorders appearing after a medium to long period.

Food poisoning: A set of symptoms after ingestion of a quantity of a toxin present in the food product, the product is dangerous to consume, even if the pathogenic microorganism is no longer alive in the product. This is the case for example of *Staphylococcus aureus, Clostridium botulinum (Botulism), Aspergillus flavus, Penicillium citrinum*.

This food poisoning manifests itself through diarrhea, vomiting, abdominal pain, neurological signs, but it is without fever and the disorders appear quickly.

2.2.2. Technological (market) quality

The technological (marketable) quality of a food product is the suitability of this product for processing and distribution. Since the consumer is not the only user, and quality is the satisfaction of all users (manufacturer and distributors), the food product must be able to survive throughout the distribution chain. The alteration of its marketable quality modifies its plastic and organoleptic characteristics and makes it unmarketable.

This alteration occurs:

- When the technology implemented to ensure the microbiological stability of the food product is faulty. Example: development of osmophilic yeasts (swelling) in a sweet product with low water activity, if the latter has not been

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perfectly controlled.

- Slowly during storage.

Microbiological control of technological quality aims to detect the presence of microorganisms that can alter the market quality of the finished product, and to verify the effectiveness of the technology after their application, in order to store and market microbiologically stable food products.

3. Microbiological control policy:

For many years, the control of this quality consisted of checking the safety of finished products, that is, their bacteriological and chemical compliance with the legislation. This examination was carried out by the manufacturer before distribution, and, eventually, by official control laboratories at the retail level.

This control of finished products had the major disadvantage of requiring waiting for the results of the analyses before being able to intervene on the production line, which resulted in an additional cost. It became desirable to be able to anticipate possible unsatisfactory results by a more suitable process, or to intervene on the process by making corrections upstream of the finished product. It was therefore necessary to carry out controls during manufacturing. Food production industries therefore began to develop a quality system to ensure a finished product that complied with the quality defined for this product by the company itself: depending on the quality it wanted for the product it manufactured, it designed and implemented its manufacturing process by referring to the quality system it had itself established.

Today, for companies, the term quality is therefore used with a different meaning, it means: ensuring the conformity of a product or service with what was planned. The quality system has also been extended to analysis laboratories and concerns the operation and results provided by these laboratories. The aim is to ensure that the manufacturing or services actually carried out by the company are in all respects compliant with what was chosen and described by the processing industry or the analysis and testing laboratory.

These analyses have often been the preserve of specialized laboratories because it is well known that the evaluation of the hygienic quality of our foodstuffs requires a lot of equipment, qualified personnel and remains cumbersome, long and costly. As a result, this

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evaluation is still difficult to apply to a number of samples representative of a batch or production.

Controlling microbiological quality (desired by the manufacturer but also the consumer) involves a set of steps ranging from the control of raw materials, during processing or the finished food, to good manufacturing practices and the identification of the main critical points of the production/distribution system, most often through a HACCP approach. These analyses are now widely used in most factories and distribution networks and allow, through the performance of judicious checks, a good assessment of quality and the identification of possible contamination, the resulting corrective actions without increasing the costs too much.

This is the objective of the HACCP (Hazard Analysis Critical Control Point) approach: risk analysis for the control of critical points. This system aims to control the manufacturing of the product from the purchase of raw materials to the consumption of the product. The manufacturing process can involve up to 80 different stages and it is impossible to control them all. It is therefore a question of locating the most potentially dangerous stages in order to then be able to control them.

Risk analysis determines when there may be potential danger from deviation from a "normal" procedure (critical point). Risk assessment consists of determining the probability of an unacceptable consequence of this deviation. Technical knowledge must therefore be used. A hazard is considered unacceptable if it allows the growth and survival of a pathogenic organism or contamination by such an organism or if it induces the production or persistence of microbial toxins in the food product or its environment.

This risk analysis leads to the identification of critical points to be controlled and mastered. A critical point is a place, a practice or a process whose factors can be controlled in order to reduce potential risks.

HACCP (Hazard Analysis Critical Control Point):

The HACCP system includes:

- Risk analysis: identification of risks and assessment of their severity (example: contamination by Salmonella during the manufacture of powdered milk);

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- The determination of critical points or controls are necessary to control the identified risks (example: contamination of fresh milk, contamination during storage, contamination during transport to the factory);

- The specification of indicative criteria for the effectiveness of the control allowing the control of the risk and the tolerance limits. A criterion is defined as a limit (of a physical, chemical or biological nature) or a specific characteristic (example: absence of Salmonella in 100 ml);

- Execution of corrective actions when the criteria are not met.

The application of a HACCP approach makes it possible to integrate hygiene into a quality approach. The results of a HACCP study are specific to the products and the type of production chain.

3.1. Level of control and parameters to be controlled:

3.1.1 Control of raw materials

This self-monitoring carried out by the company must make it possible to verify the general level of contamination and the presence of specific microorganisms likely to hinder manufacturing or alter the finished product when they are not destroyed during manufacturing (cooking, salting, etc.). The microbiological quality of the raw materials must therefore comply with the specifications. This may be different for a transformation involving fermentation; in this case, a slightly contaminated environment can be tolerated.

3.1.2. Self-inspection during manufacturing

The objective sought here is to control the manufacturing process from a microbiological point of view in order to better control it. It is therefore necessary to locate the points in the chain where there is the greatest risk of contamination. This analysis of critical points is part of the HACCP study conducted for the entire manufacturing process. It must make it possible to quickly highlight a manufacturing problem in order to be able to modify part of the process and improve the results of the analysis at the <critical point>. The result of an analysis may be higher than the set point (acceptable limit for this parameter: < 100 coliforms/mL, for example). In this case, a corrective action is triggered upstream at the level of the production chain (improvement of the cleaning and disinfection of a manufacturing accessory, for example). The effectiveness of the corrective action is verified by obtaining a result that has become satisfactory for the chosen parameter (result < 100 coliforms/mL). Self-checks must be rapid and as inexpensive as possible so that they can be carried out frequently.

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In addition, a category of microorganism must be tested that represents a good indicator of the risk of contamination at this precise stage for the product in question. It is the high frequency of self-checks that makes it possible to detect a failure in the production system as early as possible.

3.1.3. Control of the finished product

It is a control carried out a posteriori to evaluate the quality of a product after its manufacture and before its distribution.

As a self-check, it is commissioned by the manufacturer to validate production and release stocks for distribution. It can only have a limited impact on the production line because the results often require at least 24 to 48 hours. When, during this final check, a failure leading to the removal of the defective product is detected, the modification of the production line then results in intervention times on the manufacture of the product that are much longer than during a self-check during manufacture.

Routine official controls are carried out systematically to verify the conformity of manufactured products with official microbiological criteria in order to protect the consumer. This is a control of a repressive nature.

3.1.4. Control of sourdoughs

When a sourdough is used for manufacturing, its quality is checked before seeding the fermentation tank. We seek to detect a contaminant, even if present in small quantities because, after seeding the tank, it would be likely to multiply more quickly than the selected ferment. Yeasts can be contaminated by wild yeasts or lactic or acetic bacteria; molds by bacteria; lactic sourdoughs by bacteria with faster development or by bacteriophages.

3.1.5. Control of hygiene of premises and personnel

The manufacturing conditions themselves can be checked. This concerns the premises, the very design of which must ensure good hygiene conditions (surface control, ambient air control). Carried out at regular intervals, these checks can lead to changes in the system set up as part of quality assurance.

Manufacturing equipment must be designed to avoid areas of microbial proliferation (dead spots). Finally, personnel, a major source of contamination, must adhere to very strict hygiene rules.

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3.2. Frequency of control:

The frequency of analysis depends rather on the type of product. Products not subject to sanitizing during their manufacture and whose consumption is intended to be done in their state are controlled by microbiological analysis once a week.

The frequency of analysis of other product ranges whose consumption is estimated to be lower risk (sanitizer during manufacturing, expected consumption after cooking) is even lower:

•Once every two months for each range of products with sanitizing treatment and whose consumption is done after cooking;

BN• Once a year for each range of heat-treated products in their final packaging.

The frequency of checks is also determined by the production capacity of the plant and the level or fluctuation of the contamination level. These frequencies are defined by regulatory texts specific to each country and each product. (ISO 9001:2000)

Example 1

In the case of public water supply, the frequency is linked to the number of inhabitants. The WHO recommends taking samples at the entrance to the network according to the following frequencies:

- ✓ Less than 20,000 inhabitants at least every month.
- ✓ Between 20,000 and 50,000 inhabitants at least every 15 days.
- ✓ Between 50,000 and 100,000 inhabitants at least every 4 days.
- ✓ More than 100,000 inhabitants every day

Note: If the water is treated, samples must be taken every day before and after treatment. In all cases, the frequency must be adapted to the risk and the volume of water collected.

Example 2

Case of dairies: the official hygienic control of the processing workshops provides for the control of milk according to the quantity of milk processed and according to the following frequencies:

- ✓ 2 times per day quantity of milk treated ≤ 5000 l/d
- ✓ 3 times per day quantity of milk treated between 5000 and 10,000l /:

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- ✓ 5 times per day quantity of milk processed ≥10,000 l/d

Samples should be taken at time intervals greater than 15 min.

3.3. Control methods:

The analysis methods implemented must be rapid, reliable, reproducible and if possible simple (and inexpensive); they consist of a search for and/or a count of the main microbial germs found in our foods in order to control their presence or absence (in the case of dangerous germs responsible for infectious diseases) and their number (in the case of less dangerous germs, contaminants or normal hosts of the raw materials making up the foodstuff).

(never lose sight of the fact that it is absolutely necessary to guarantee consumer safety by allowing the detection of dangerous microorganisms or possibly toxic metabolites or microbial toxins and ensuring good product quality and good conservation).

From a health perspective, the list of "dangerous" bacteria responsible for illnesses following consumption of contaminated food is tending to increase: in addition to various *Salmonella, Shigella, enterotoxigenic Staphylococcus aureus* and *Clostridium perfringens, Bacillus cereus, Vibrio parahaemolyticus, enteropathogenic Escherichia coli* and bacteria of the genera Streptococcus, Listeria, Campylobacter, Yersinia, Brucella, etc. have been added.

Routine microbiological control of a solid or liquid food product most often consists, in the absence of information on the possible involvement of this product in an infectious disease, toxic infection or intoxication, of:

- A sterility check for products subjected to antimicrobial stabilization treatments (temperature, additives, etc.).
- An estimate of the number of contaminants (total mesophilic aerobic flora, coliforms, sulfite-reducing anaerobes) or their detection - identification (Salmonella, Listeria, etc.).

This check is currently long (several days), which often involves:

- To store the product while waiting for the response (impossible for highly perishable products)

- To distribute the product without knowing its bacteriological quality with all the risks that this entails.

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4. Collection, transport and preparation of samples

4.1. Conditions of collection

The essential conditions to be respected for sampling are first of all compliance with the rules of asepsis (correct work of the microbiologist) and the non-modification of the flora present in the product. As far as possible, the samples of the product to be analyzed must be brought to the laboratory in their original packaging, which avoids certain contaminations. If the product is presented in the form of large volumes (milk tanks, etc.), ensure the good homogeneity of the distribution of microorganisms; a representative part of the product will be taken sterilely. It is sometimes necessary to take samples at various levels of the food (surface, depth of a solid food) or after grinding and homogenization. The manipulations carried out during sampling must in no case cause contamination: need to use sterile instruments and work sterilely. Some instruments must be sterilized at the place of sampling. Soaking in alcohol and flambéing are sometimes insufficient because the temperature reached is not high enough. It is necessary to use clean, dry, airtight, wide-necked bottles sterilized in a Pasteur oven (160°C - 10 min) or by autoclaving at 121°C for 30 min or single-use and sterile; their size must be adapted to the volume of the sample. The containers can be made of glass, metal or plastic (polyethylene, polycarbonate, polypropylene); in the latter case they are single-use containers whose sterilization is obtained cold (radiation). In all cases, the sampling containers must have a hermetic closure system. The sampling of a packaged product must be carried out in the sterility zone of a bunsen burner or an equivalent system.

4.2. General procedure for food sampling

Once the sampling plan has been determined, the material prepared and the sampling site identified, proceed to collect the food aseptically.

At all times, unless otherwise specified, take a minimum of 300 g or ml. It is important to consider the physical state of the product (liquid, solid) and its storage temperature (room temperature, refrigeration or freezing). If this minimum cannot be reached, note this on the analysis request. For bulk products, the 300 g must be taken from approximately five different locations in the batch or container.

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4.2.1. Surface sampling

- **Swabbing**

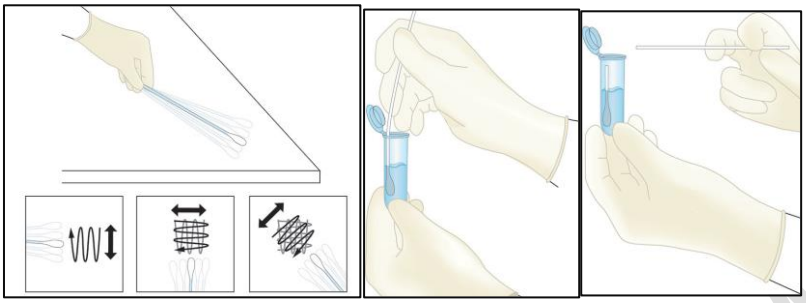

Figure 02: Surface swabbing technique

A cotton swab is immersed in sterile Ringer's solution at 1/4 or in tryptone-salt broth with added Tween (0.5°/°°). The sample is taken by rubbing on the surface of the product; the swab is then immersed in 10 ml of Ringer's at 1/4 or 10 ml of tryptone-salt broth. The analysis is carried out from the suspension thus obtained.

- Rinsing: This method is used in the case of containers or pipes; a known volume of sterile solution is introduced into the material to be analyzed. After stirring, the liquid is collected and subjected to analysis.

- Fingerprint method: a previously UV-sterilized adhesive tape is applied to the surface to be studied. After a few seconds of contact it is removed and applied to the surface of a suitable agar medium. After a few hours of contact at the desired incubation temperature it is removed and the dish is incubated until colonies appear.

This technique can also be replaced by the use of gelatin slides ready to be placed in direct contact with the surface to be analyzed, and incubated directly. (Figure 03).

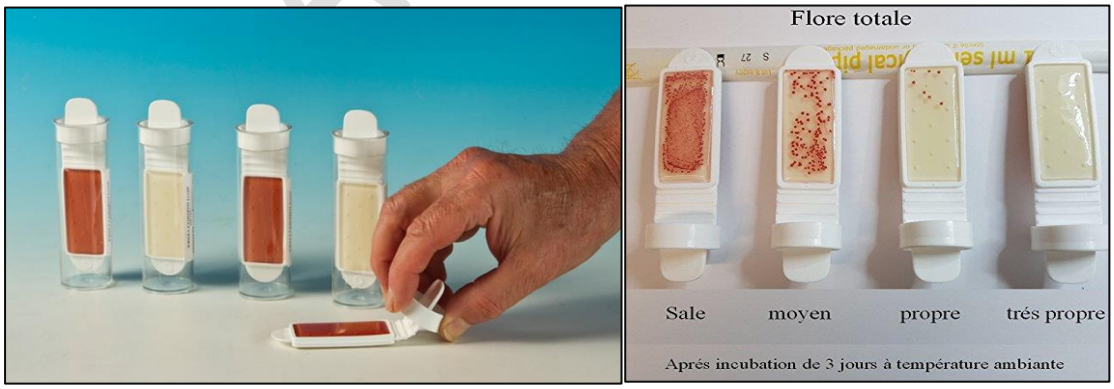

Figure 03: Agar slides

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4.2.2. Collection of liquid products

The technique varies with the product, the volume and the shape of the container. However, it is always necessary to ensure that the liquid is perfectly homogenized (stirrers) before taking the volume required for analysis using a pipette (or a sterile tested bottle or other).

4.2.3. Collection of solid products

Depending on the product, the sample will be taken with a scalpel, a probe (cheeses and soft products) or a harpoon pipette. The surface is often removed before taking the sample. If the product is heterogeneous (prepared meals, preserves, etc.), it is necessary to ensure that the sample is representative.

4.2.4. Frozen products

Use sharp instruments such as: knife, probe or sterilized wick, if required. For microbiological tests, unless otherwise specified, frozen samples may arrive at the laboratory thawed provided that the temperature is equal to or less than 4oC during thawing. Thawing may be done during transport. Thawing and refreezing should be avoided for microbiological tests. Dry ice should only be used when the frozen state is necessary for the validity of the result.

4.2.5. Original containers - Finished products

Whenever possible, when sampling units are small, collect the original closed containers to minimize the risk of contamination. Containers should be placed in clean plastic bags if their seal can be easily compromised (e.g. all products wrapped only with Saran wrap). Open, broken or damaged containers should not be sampled.

4.3. Sample processing

4.3.1. Transfer of the sample to the laboratory

When the aseptic sampling has been carried out, the product must be immediately identified with a label or reference. It is advisable not to use felt-tip pens on plastic films (PVC) because the ink can penetrate and disrupt the analysis. Note the initial temperature, the time of sampling, the date and the transport temperature. Then bring the samples to the laboratory as quickly as possible, maintaining the initial conditions in which the product was. The analysis should be carried out within one hour of sampling.

Upon receipt at the laboratory, the sample accompanied by its data sheet is recorded.

If the sample is to be transported, the time before analysis should be kept to a minimum. It is often necessary to refrigerate (but not freeze) the product during transport;

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some fragile germs may disappear during this refrigeration. If a product is dehydrated or canned, it should not be refrigerated.

For a frozen product, ensure that there is no thawing during transport (this product can be kept for 1 month before being analyzed). Freezing a product causes a more or less significant reduction in the number of germs it contains. It is necessary to ensure that the temperature of the product taken is at least -18°C, transport the product at this temperature and thaw in ambient air at a temperature of around 20°C for less than 3 hours, sufficient time to reach a texture that allows sampling. (nature, date, time, source of sampling, name of the sampler, analyses requested, other useful information).

4.3.2. Sample preparation

Whatever the initial nature of the product, microbiological analysis is always carried out from a suspension. After aseptic opening, the sample will be "homogenized" (liquid) or crushed in a known volume of sterile diluent (solid), which in fact constitutes the first dilution. For liquid (or semi-liquid) products, vigorous manual stirring in the presence of glass beads allows satisfactory homogeneity to be obtained.

For solid products, various "grinding" techniques can be used:

1) manual grindingl in Potter or in the presence of sterile sand or glass beads (mortar)

2) mechanical grinding with an electric knife mill of the VIRTIS type. During grinding, the germs must be dispersed but not destroyed; the temperature must not rise too high (the container can be placed in ice). Grinding is generally carried out with 10 volumes (or 9) of diluent for 1 "volume" of product (for example 10 g of product and 100 ml (or 90) of sterile diluent). The diluent can be distilled water, physiological water, Ringer 1/4 or a tryptone-salt solution, etc.

- with a STOMACHER type grinder. This device allows the food to be dispersed in the diluent under relatively gentle conditions. In addition, it is not necessary, as in the case of using a Virtis type grinder, to sterilize the containers between two uses. In fact, the Stomacher (digester) uses sterile single-use plastic bags. The test sample is most often 10 g (or 25) of food and 90 ml (or 250) of diluent.

4.4. Dilution techniques

During sample preparation (and dilutions) microorganisms can be inhibited or even altered by the change in the medium linked to the addition of diluent (change in pH but

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especially in ionic strength). The bactericidal effect of certain diluents is known: thus Staphylococcus aureus is "killed" in a few hours in distilled water as are most enterobacteria (E.coli); the same is true for Streptococcus pyogenes in physiological serum or in Ringer at 1/4 and for Escherichia coli in salt water at 8.5‰. Currently it seems desirable, unless otherwise indicated for a given type of food, to carry out the preparations and dilutions of the samples to be analyzed in a solution of typotone salt. In the absence of information, the choice will be made according to the composition of the product to be analyzed: if the product is rich in protein and minerals, Ringer or physiological water are sufficient and even a 2% phosphate solution and pH 7.4 in the case of fresh cheeses, fresh creams and caseinates.

Table 01: The different types of diluents used

tryptone sel	**Ringer (solution mère)**	**Eau physiologique**	**Eau peptonée tamponnée**
tryptone 1 g NaCl 8,5 g eau D 1000 ml pH = 7	NaCl 9 g KCl 0,42 g $CaCl_2$ 0,48 g $NaHCO_3$ 0,2 g eauD 1000 ml	NaCl 9 g eau D 1000 ml	bacto peptone 20 g NaCl 5 g $Na_2 HPO_4$ 9 g $K H_2 PO_4$ 1,5 g eau D 1000 ml pH = 7,2

All these diluents are sterilized at 121°C for 20 minutes.

It should be noted that tryptone-salt and buffered peptone water media allow, under specific conditions (temperature, time), revival to be carried out. Buffered peptone water is used with certain acidic products with a high buffering capacity such as yogurts.

Dilutions require the presence of numerous test tubes most often containing 9 ml of sterile diluent and numerous sterile pipettes of 1 and 10 ml. The pipettes can be replaced by automatic pipetting systems equipped with single-use cones. All manipulations must be carried out with all the aseptic precautions required in microbiology. The possible introduction of a contaminant or contamination of the operator must never occur. The container containing the liquid to be diluted is shaken manually with care to avoid splashing for about ten seconds. 1 ml of this liquid is taken sterilely (aspirated and discharged once before sampling) and

placed in a tube containing 9 ml of sterile diluent. The tube is shaken by rotating movements or by means of a Vortex. This gives a 1/10 dilution. With a new 1 ml pipette, 1 ml of this dilution is taken and placed in a new 9 ml tube of diluent; we obtain a dilution of 1/100 and so on until the desired dilution level.

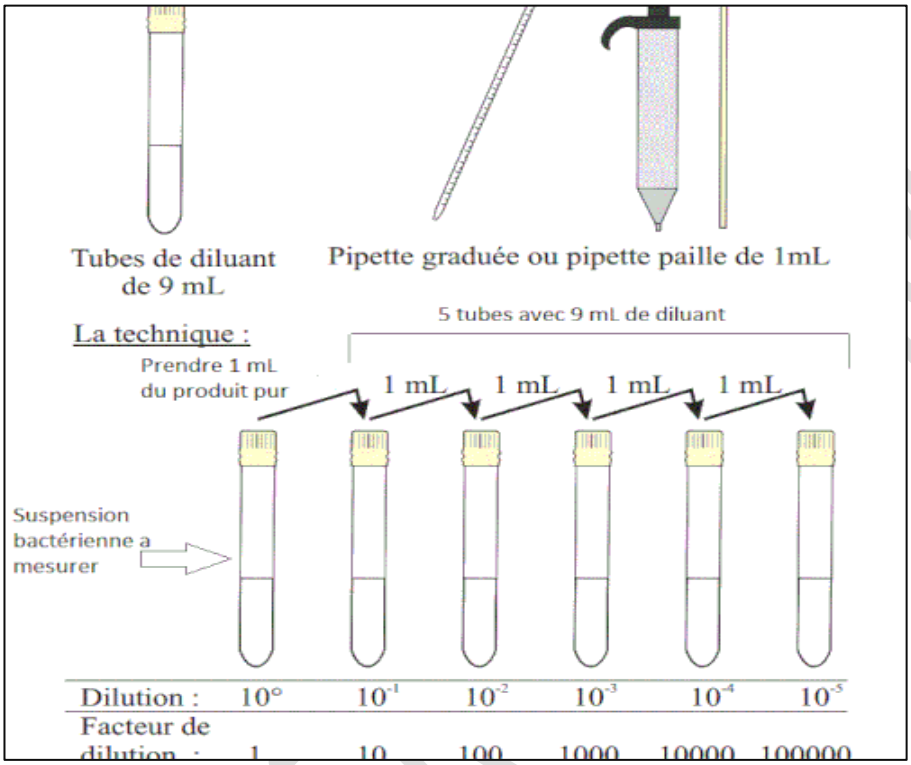


Figure 04 Dilution technique

4.5. Revival

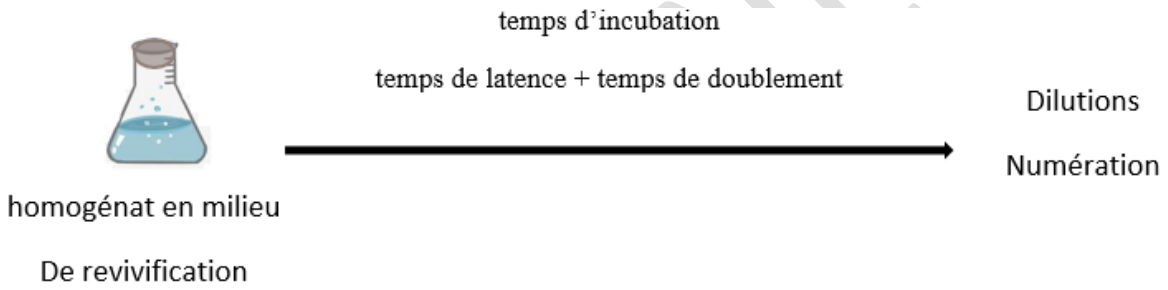
Microorganisms are often “damaged” but not killed during technological treatments (dehydration, heat, cold, etc.) applied to food products or as a result of their aging. These alterations are reflected in some of their physiological properties, in particular in their increased latency phase or their nutritional needs or in their sensitivity to unfavorable environmental conditions (pH, bile salts, dyes, salts, etc.). In general, these alterations are reversible and after their disappearance, the bacteria recover their initial properties, in particular in terms of their growth or pathogenicity. The need to facilitate the “recovery” of cells that have undergone sub-lethal alterations, i.e. their “resuscitation” or even their revival, is essential before subjecting them to selective environments that are often unfavorable to growth due to the presence of inhibitors.

Indeed, the presence of damaged cells can cause variations in counts or lead to the belief that there are no or few germs and therefore no or no risk to the consumer. This is

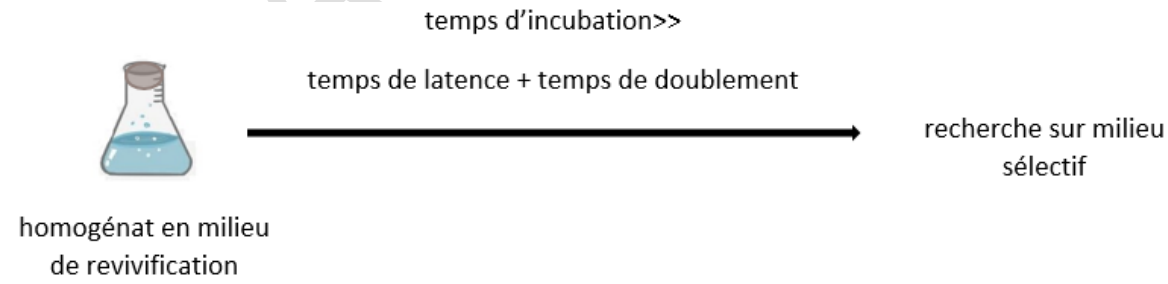
particularly important when determining whether or not pathogenic or indicator microorganisms are present.

4.5.1. Revivification in liquid medium

Revivification can be carried out from the first stage of the analysis during which the product is added with diluent. It is then sufficient to choose a diluent of favorable composition and to incubate the whole at the optimal growth temperature of the germ to be sought for a time which will vary according to the type of analysis carried out, time which is most often close to the latency time of the "normal" germ. If the revivification time is greater than the latency time, there will be a multiplication of unaltered germs which will lead, depending on the counting techniques used subsequently, to an overestimation of the number of germs. After this preliminary revivification phase, counting can be carried out either in liquid medium or in solid medium.

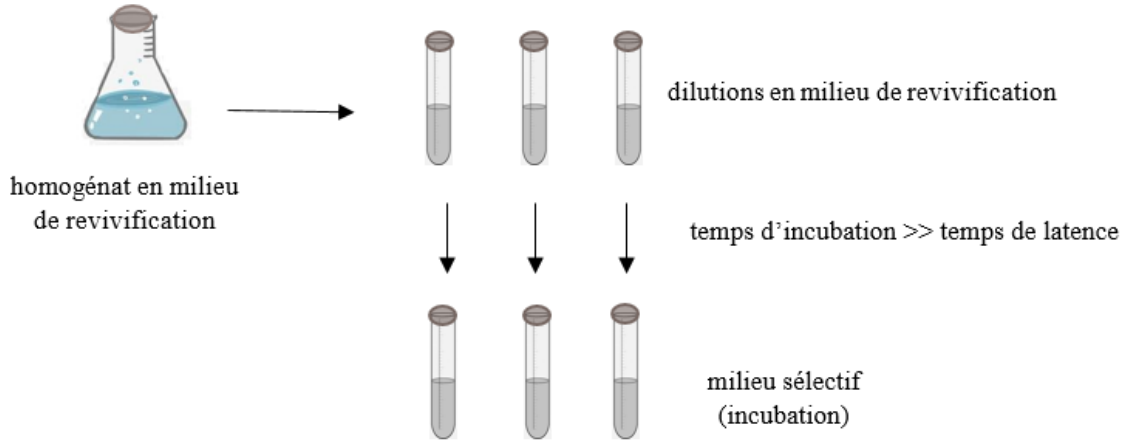


In presence or absence tests, revivification is obtained by pre-enrichment in a favorable medium (e.g. Salmonella detection). The incubation period can be relatively long because the multiplication of the germs to be detected is in this case very desirable.



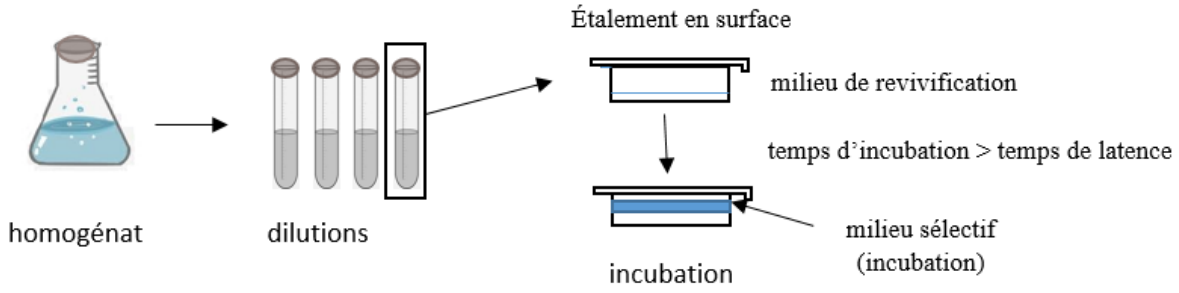
In the case where germ count must be carried out, two other methods are still possible:

- When the count is carried out in liquid medium, revivification can be carried out in non-selective media inoculated from the different dilutions. It is from these media that the selective media are then inoculated (from tube to tube).



4.5.2. Revivification on solid medium

- When the count is carried out on solid medium, revival can be obtained by pre-culture on a favorable non-selective agar with an incubation time greater than the latency time, but not allowing the formation of visible macroscopic colonies. After revival, the surface of the agar is covered with selective agar.



-When the count is carried out after membrane filtration, the membrane on which the germs are located is first placed on the surface of a revival medium for a time which may allow several normal cell divisions, then the filter is placed on the surface of a selective medium. As an example, the optimal conditions for revival of "stressed" germs by this method are as follows:

Table 02: Method of reviving some bacterial genera

Treatment	Germ	Revival	
		Time h	T
Heat	Coliforms	5	35
Freezing	Coliforms	4	35
	Enterococci	4.5	35
Acid	Coliforms	4	35
Drying	Coliforms	2	25
	Enterococci	4	25

5. Main counting techniques

In food microbiology, the interest in both the quantitative and qualitative study of the flora present in a food is considerable. Although many counting techniques can be used, there is currently no perfect technique. Some methods do not allow to differentiate between living and dead germs, others are unable to count microbial cells individually when they are associated (Staphylococcus, Streptococcus, mycelium, etc.) and allow to evaluate colony forming units (CFU) or turbidity forming units (TFU).

5.1. General direct methods

5.1.1. Direct counting

It is carried out under a microscope using graduated microchambers of known volume (Thoma cells, Malassez cells, etc.) after dilution in physiological saline for dead germs, in 10% formalin water for living germs. This type of counting can be automated (particle counters). It is also possible to estimate under a microscope the number of microorganisms in a given product after spreading and staining a known quantity of the product (of the order of a few microliters) on a slide. Finally, a direct count can be carried out after filtration of the product (or its dilutions) on a membrane, staining of the membrane and microscopic observation. This last technique allows the evaluation, after staining with acridine orange and observation in epifluorescence, of living and dead microorganisms (DEFT). This dye is an intercalator of nucleic acids with which it forms green fluorescent complexes (DNA) or orange (RNA)

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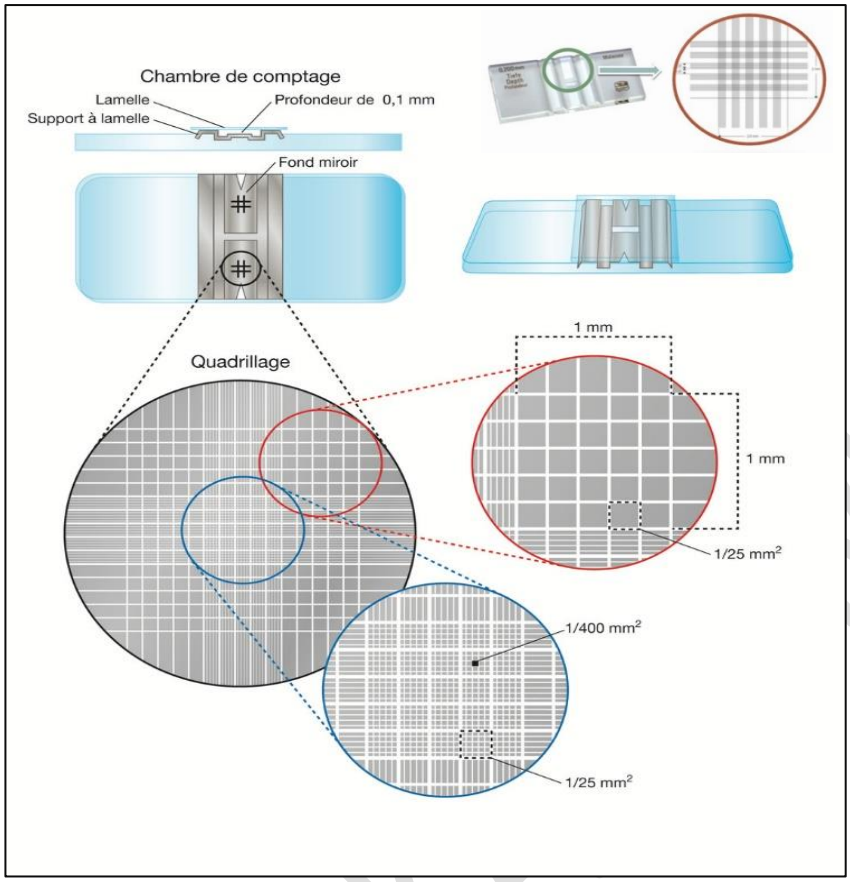

Figure 05: Counting cell

5.1.2. Determination of dry weight, or microbial protein assay or nucleic acid assay

5.1.3. Nephelometry

The turbidity of a medium is, under certain conditions, proportional to the number of microorganisms present in this medium. It is also possible to visually compare the turbidity of the medium with a standard range of albumin (Mestrezat method: thus the opacity of a suspension of 5.109 staphylococci per ml is identical to that of an albumin suspension at 0.5 g per liter).

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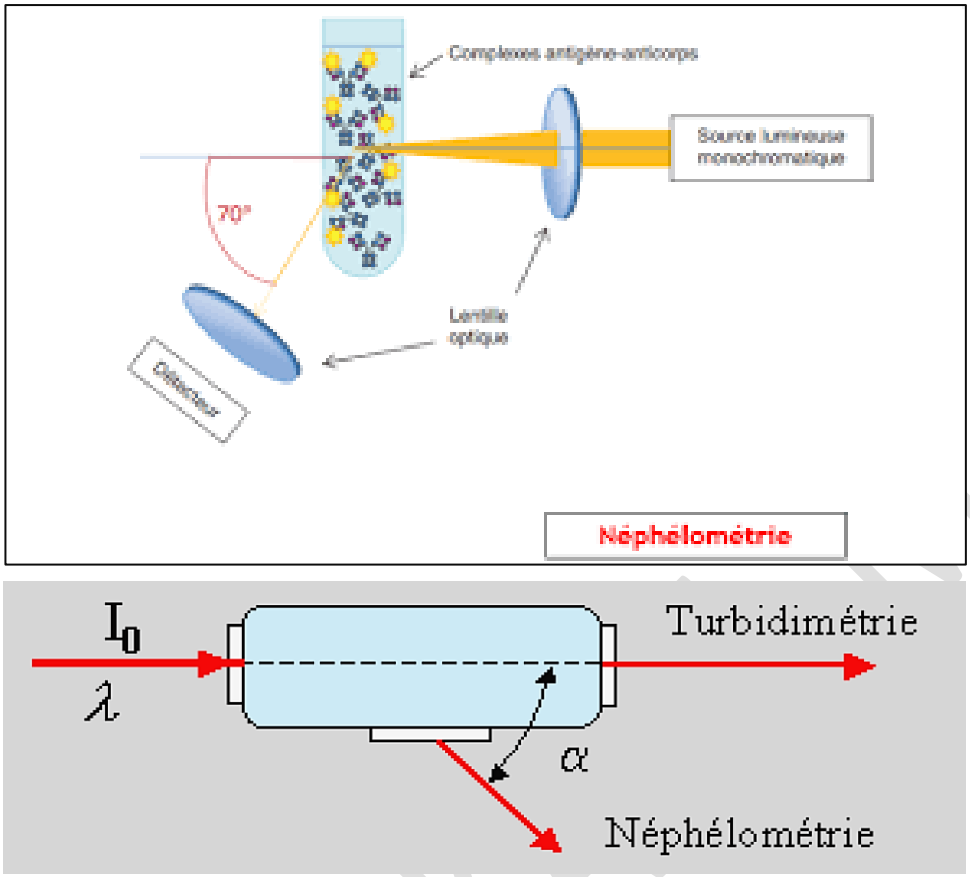

Figure 06: principle of nephelometry

5.1.4. Luciferin-luciferase system

This method allows the counting of living microorganisms by measuring intracellular ATP, the intracellular ATP content being substantially constant for a given microorganism; this content is equal to 0 if the germ is dead.

5.2. General indirect methods

5.2.1. Assay of a synthesized primary metabolite, or assay of the quantity of a substrate consumed.

These methods are only applicable under well-defined conditions (exponential growth phase, temperature, environment, etc.).

5.2.2. Reduction of a dye

Some microorganisms modify the oxidation-reduction potential of the environments in which they are found; the speed of this modification is a function of the number of microorganisms, their metabolic activity (most often reductive) and other parameters such as the nature of the environment, the germ, the temperature, etc.

Among the available indicators, methylene blue and resazurin are the most used.

5.2.3. Dilution and culture

This technique allows in principle the counting of living germs. The culture is carried out either in liquid medium (1 germ or a group of germs gives after inoculation and incubation 1 positive culture) or in solid medium (1 germ or a group of germs gives rise to a colony). In the latter case the inoculation can be done in the mass of the agar or on the surface.

5.2.3.1. Counting from solid medium: CFU

This methodology is most frequently carried out in Petri dishes. It is based on the principle that any living bacteria introduced into the mass or on the surface of a favorable agar medium in principle gives rise after incubation to a macroscopic colony. The total number of colonies then corresponds to the number of CFU present in the inoculum. Many criticisms of this method can be made. Thus, strict aerobic bacteria develop poorly in the mass of the agar, while strict anaerobic bacteria will only develop there under appropriate incubation conditions (anaerobic jar for example). There may also be certain bacterial antagonisms (bacteriocins etc.). Furthermore, if the mixing temperature of the agar medium mixed with the inoculum is higher than 45 - 47 ° C, there may be inactivation of microorganisms. This method of culture in the mass nevertheless leads to homogeneous dispersions in the agar and therefore to a homogeneous distribution of the colonies. In this method, germs will be found in the mass and others on the surface, the colonies formed being, given the steric constraints imposed by the gelled network, then different for the same microorganism. This disadvantage is eliminated by the double layer technique.

Surface counting can only be carried out on a perfectly dry medium surface. The spreading operation with a rake must be mastered (adsorption of germs on the rake) and the volume of inoculum deposited cannot under any circumstances exceed 0.5 ml with 9 cm diameter dishes (larger volumes are not absorbed by the agar gel and the water remaining on the surface makes any counting impossible due to the formation of a layer). This method gives good results with aerobic and aerobic-anaerobic germs.

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Figure 07: Different counting methods

a. Agar mass counting technique

The agar media (distributed in Erlenmeyer flasks or 15 ml bottles) are liquefied in a boiling water bath or, better, in a microwave oven, then kept supercooled in a water bath at 45 ± 1 ° C. 1 ml of the liquid in which the number of microorganisms is to be determined is introduced into the center of the Petri dish placed flat in the Bunsen burner protection zone. The inoculum can be distributed in drops on the bottom of the dish. In order to use only one sterile pipette for all these operations, it is recommended to start the inoculation with the highest dilution and finish with the undiluted liquid.

Carefully note on each box the origin of the analysis, the medium used and the corresponding dilution (on the side so as not to be hindered later for counting).

The procedure is the same for each dilution, carrying out, where possible, two tests per dilution. The supercooled agar medium in which the inoculum will be incorporated must be at 45°C±1°C. If its temperature is higher than this value, partial destruction of the flora will occur; on the contrary, if its temperature is lower than 45°C, the medium will solidify irregularly and will not mix homogeneously with the inoculum. To introduce the agar medium: remove the medium from the 45°C water bath, wipe the container, open it aseptically, flame its opening and pour the medium into the Petri dish containing the inoculum after having opened it ajar in the sterile area. Do not press the container on the dish. Mix quickly by circular and alternating horizontal shaking; avoid sudden movements which risk projecting the inoculated medium onto the edges or even outside the dish. Leave the cans to cool flat until completely solidified (about 30 minutes). Turn the cans over and place them in the oven in this position at the required temperature.

To avoid the formation of large colonies on the surface (compared to those that will develop in the mass of the agar), a thin layer of culture medium identical to that already present in the dish can be poured onto the surface of the inoculated agar or a thin layer of non-nutritive agar can be poured onto the surface (double layer technique). (Figure 08)

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b. Agar surface counting technique

100 to 500 microliters (graduated pipette or better automatic pipette) of the medium to be analyzed are deposited on the surface of the agar and immediately distributed uniformly on the surface of the medium using a sterile inoculator of the rake pipette type. The rake pipette is "sterilized" between two spreadings by immersion in ethanol. The ethanol adsorbed on the glass is then ignited (this operation does not deform the inoculator).

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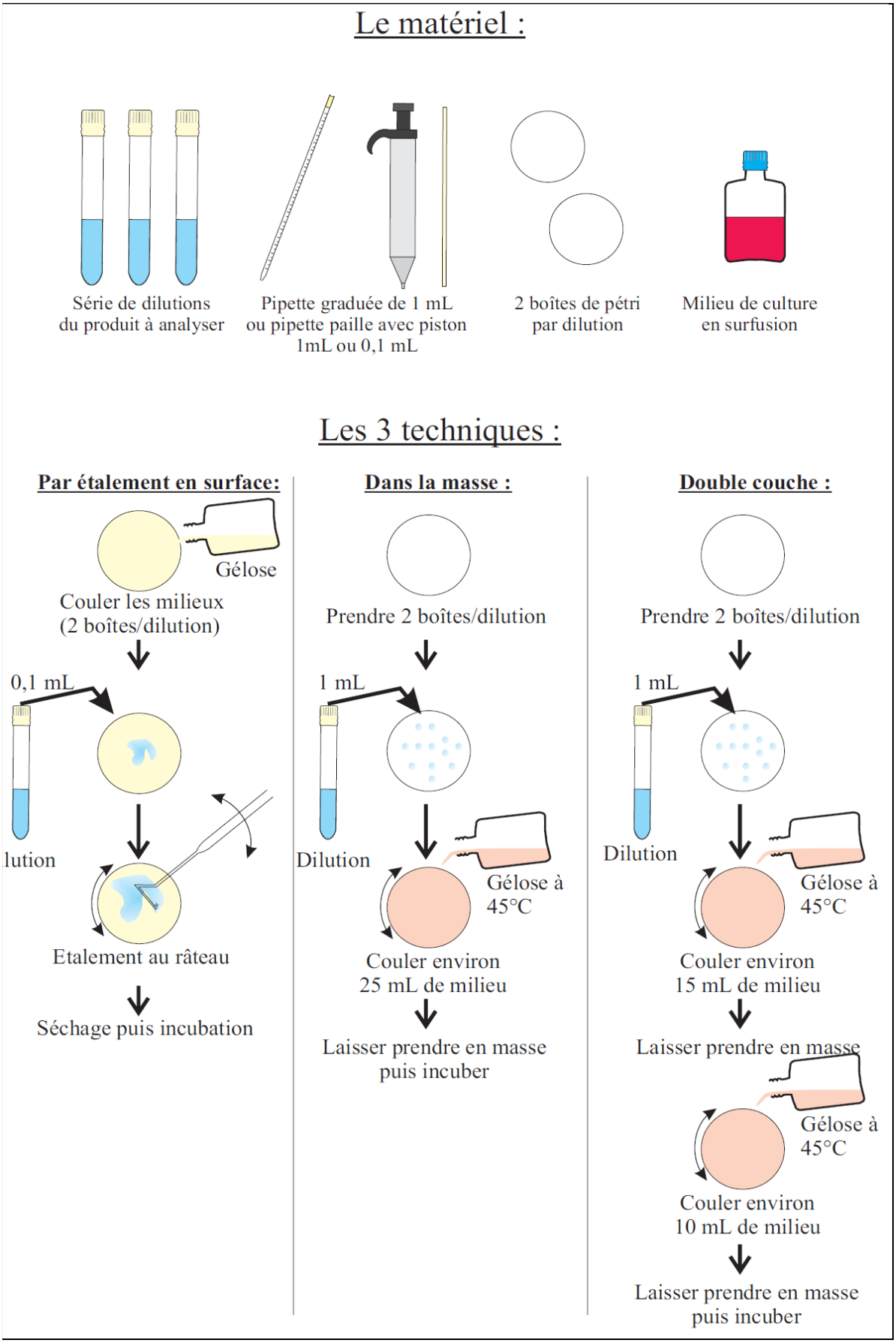

Figure 08: Solid medium counting techniques

5.2.3.2. Liquid medium counting: UFT

This method has certain advantages such as the possibility of studying a biochemical characteristic of the germ that is difficult to highlight on agar medium such as gas production (bell) or of easily carrying out the count with a revivification phase. This method is based on the fact that an inoculum containing at least 1 germ (UFT) will give, after introduction into a given liquid medium, a positive culture. In this technique, the availability of nutrients for the microorganism is excellent.

a. fractionation of large liquid volumes

To avoid the accumulation of degradation products that could have an inhibitory action, 1 ml of inoculum is inoculated into 100 ml of culture medium. This medium is then aseptically distributed into 10 test tubes at a rate of 10 ml per tube, then incubated. Assuming a homogeneous distribution of microorganisms, it can be concluded that there are 1 to 10 microorganisms in the initial inoculum of 1 ml.

- If all tubes grow.....more than 10 germs/ml
- If 9 tubes grow.....more than 9 germs/ml
- If 1 tube cultivates..... more than 1 germ/ml.

b. Mac Grady method (Most probable number technique MPN)

This method reveals smaller quantities of germs than most solid medium counting methods. It is based on statistical analysis and provides most probable numbers (MPN) by calculation. There are many variations to the method depending on the microbial load to be assessed. This method assumes that the microorganism is normally distributed in the medium and therefore that the same volume of sample contains on average the same number of microorganisms (in reality it contains a little more or a little less); the most probable number corresponds to the average value. If the number of microorganisms is low, the deviation from the average may be high and vice versa.

Example: a liquid product contains 100 microorganisms / 100 ml

Samples of 10 ml contain on average 10 germs with tubes at more than 10 and few at less than 1. Samples of 1 ml contain on average 1 germ with tubes at more than 1 and negative tubes.

Samples of 0.1 ml result in 1 positive tube for 10 tubes and many tubes are negative.

-Microbial load count between 0 and approximately 15 per 100 ml

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In this case 50 ml of product are introduced into an Erlenmeyer flask with 50 ml of suitable culture medium at double concentration, and 5 times 10 ml introduced into 5 tubes containing 10 ml of culture medium also at double concentration. After incubation the positive tubes are identified and the MPN for 100 ml of product is determined using the following table:

Positive Erlenmeyer flasks	**10 ml positive tubes**	**NPP/100ml**
0	0	0
0	1	1
0	2	2
0	3	4
0	4	5
0	5	7
1	0	2
1	1	3
1	2	6
1	4	9
1	5	16
1	6	+18

- Microbial load count between 0 and 100 germs per ml

In this case, 1 ml of the stock suspension and then its dilutions are introduced into tubes containing the chosen culture medium but each test is carried out in duplicate (or in triplicate or more depending on the desired level of precision).

With the test comprising two tubes inoculated by dilution, we note, after incubation, the positive or negative responses and we assign:

- the number 2 to two positive tubes seeded with the same dilution

- the number 1 if one of the two tubes gives a positive response at a given dilution

- the number 0 if the culture is negative for both tubes.

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Exemple

	produit pur		dilutions							
			10^{-1}		10^{-2}		10^{-3}		10^{-4}	
	tube 1	tube 2	tube 1	tube 2	tube 1	tube 2	tube 1	tube 2	tube 1	tube 2
résultat après incubation	+	+	+	+	+	-	-	+	-	-
affectation	1	1	1	1	1	0	0	1	0	0
nombre par dilution	2		2		1		1		0	
nombre de trois chiffres	← 221 →				← 110 →					
			← 211 →							

Adjacent digits are grouped by 3; we obtain 3-digit numbers which are 221, 211, 110. The smallest number and for which, if possible, the units digit is a 0 (110 here) is chosen. For this number, the Mac Grady table gives the most probable number of bacteria per ml or pure product and this obviously for the two-tube test by dilution. The hundreds digit (1 in the case of 110) corresponds to the dilution to be considered to give the NPN per ml of product. Thus in the chosen example 110 corresponds to the dilution "-2" which gives, according to the Mac Grady table, 1.3 bacteria (UFT) per ml. This MPN therefore corresponds to the dilution 10-2, or to an MPN of 130 bacteria (UFT) per ml of undiluted product.

There are Mac Grady tables for 3 or 5 tube tests with inoculum volumes of 1 or 10 ml and even 50 ml.

5.2.4. Counting by filtration

This method consists of passing a certain volume of sample or its dilutions through a filter membrane (for example a Millipore or Sartorius membrane or ... 47 mm in diameter and with an average porosity of 0.45 mm to 0.22 µm) on which the microorganisms sought are retained.

After filtration, the upper funnel is rinsed with sterile distilled water or with a sterile buffered solution in order to recover all the germs and to eliminate from the filter itself any microbicidal agents. The filter is then placed on the surface of a counting medium soaking a suitable filter or on an agar medium specific to the germ to be tested for, with the side bearing the microorganisms facing upwards. After incubation, as in the case of counting in agar medium, the colonies formed on the surface of the filter are counted.

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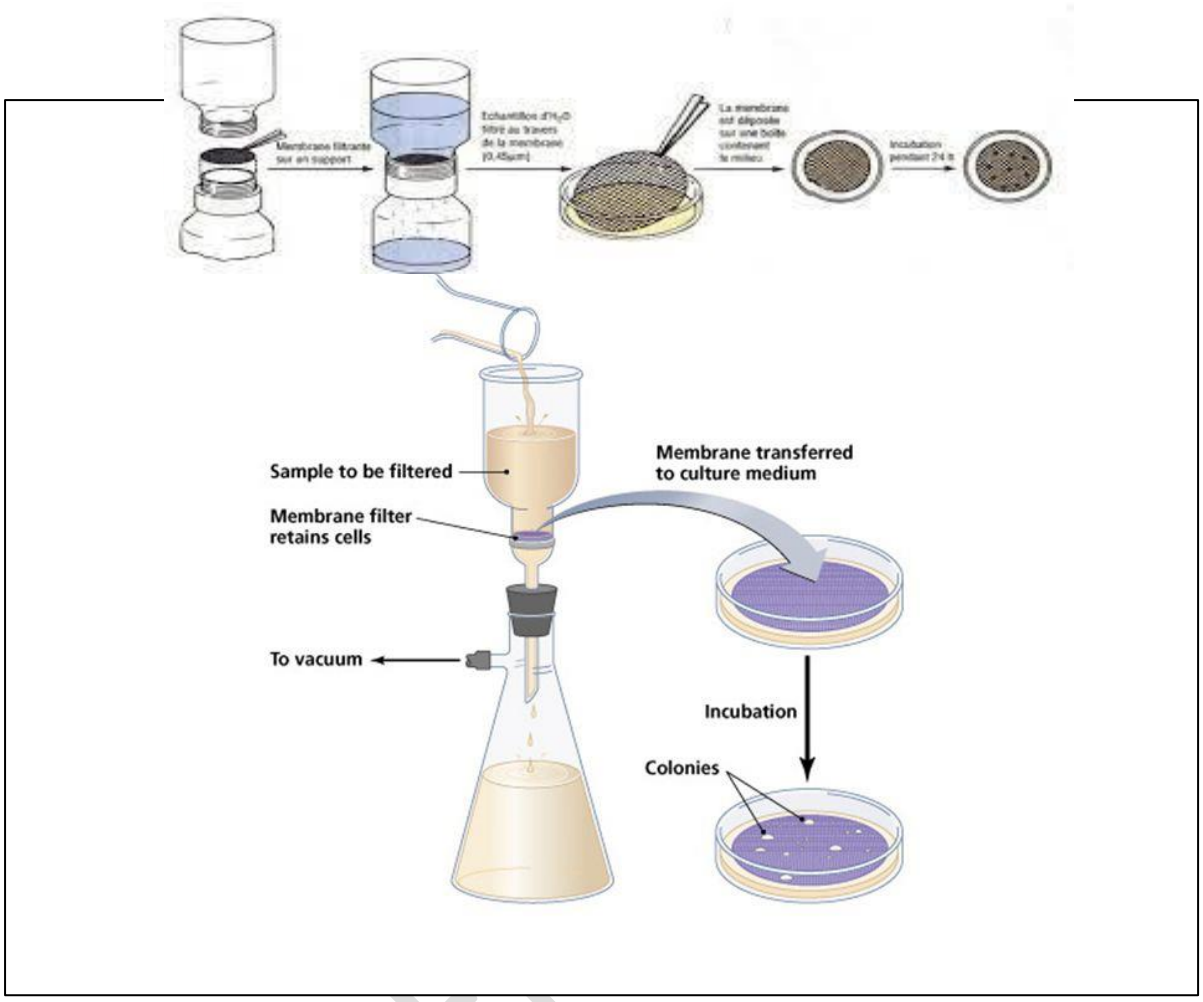

Figure 09: Filtration counting technique

This method has some advantages over the methods already written. Thus, the incubation time is generally shorter (18 hours instead of 24 hours), the volume of analysable sample is larger (up to several litres, which makes it possible to carry out germ counts in liquids containing very little such as certain waters); there is no interference between micro-organisms (separated on the surface of the filter and fed by diffusion of the culture medium through the pores) and the microbicidal agents associated with the product are eliminated (chlorine, antibiotics, preservatives etc.).

The differences between the results of the specific counts of a given microorganism most often originate from the nature of the culture medium chosen as well as the method used,

the temperature and the duration of the incubation without omitting the variations linked to the handler.

5.3. Interpretation of numbers

5.3.1. Calculation of the microbial load of the product

From the number of colony forming units or the MPN determined for a given dilution, it is necessary to "go back" to the product analyzed. In the case of a liquid product, this presents no difficulty since it is sufficient to multiply the number or the MPN found at a given dilution by the inverse of the dilution. If, for example, 256 colonies were counted in the dish inoculated from 0.1 ml of 10-3 dilution of the product, the number of CFU per ml of product is equal to:

256 x 1 / 0.1 x 1 / 10-3 or 256.104 CFU / ml.

In the case of a solid product, the initial grinding or suspension is considered a dilution that must be taken into account. For example, if 10 g of product were suspended in 90 ml of tryptone-salt broth, 1 ml of this suspension is considered to correspond to 0.1 g of product. This is an approximation because strictly speaking, the suspension should be carried out by taking a given weight of product and topping up to a specific volume with diluent. It is sometimes possible to take into account the water content of the product.

5.4. Recent counting techniques

The processes previously studied require a fairly long multiplication phase to make each cell individually accessible for observation, most often under the form of a colony visible to the naked eye.

5.4.1. Spectroscopy

5.4.1. 1. MALDI-TOF mass spectrometry

As its name suggests, mass spectrometry is concerned with measuring the mass of molecules or atoms present in a sample. The principle of this measurement is based on the possibility for a flow of ions to be deflected by an electric and/or magnetic field, the trajectories being proportional to the mass and charge of each of the ions. This principle requires the molecules and atoms of a sample to be previously transformed into ions in the gas phase, before being analyzed by a mass spectrometer.

A mass spectrometer always includes (Figure 10):

- an ionization source: passage of the sample into gas phase and ionization of the molecules

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- an analyzer: separation of ions according to their mass/charge ratio (m/z)
- a detector: it allows detection of previously sorted ions and provides an electrical signal proportional to the number of ions detected.
- a computer signal processing system to visualize spectra

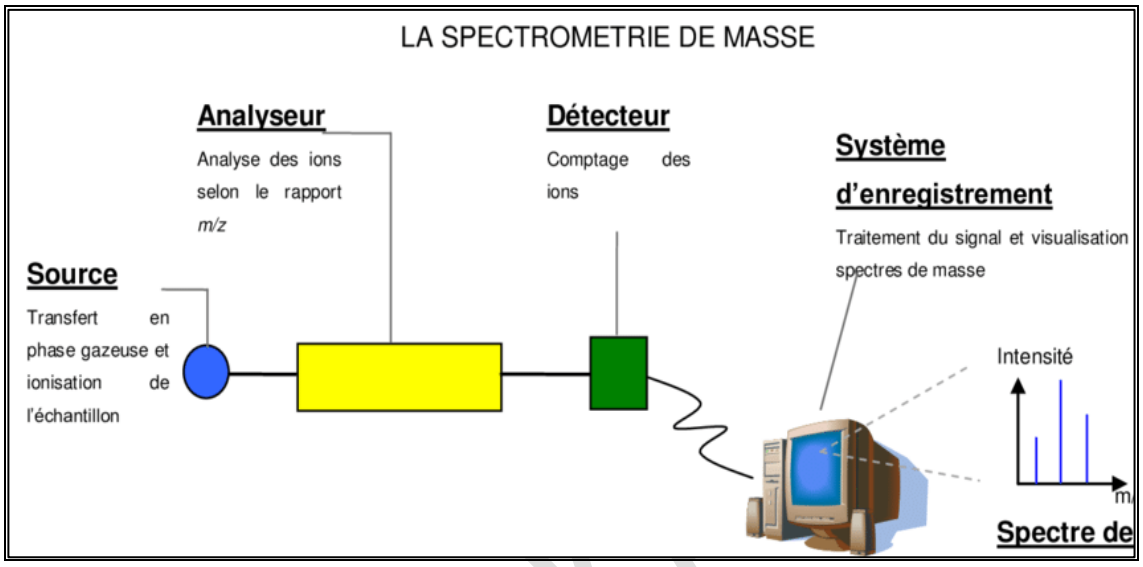

Figure 10: Principle of the Maldi-Tof technique

5.4.1. 2. Spectrophotometry

5.4.2. Measurement of variations in the redox potential

Microorganisms, by multiplying in an environment, absorb oxygen and release it reducing substances which lower its oxidation-reduction potential. The observation Visual detection of the turn delay of redox dyes such as methylene blue is a very total flora assessment employee. This observation can be done using a spectrophotometer.

5.4.3. Measurement of enzymatic activity

The principle is based on the fact that enzymes are catalytic molecules and that the measurement of enzymatic activity is based on the dosage of the products of the enzymatic reaction. cite as an example:

- **The dosage of phosphatidic activity (thermoresistant) in dairy to assess the** quality of milk intended for cheese making. This is a good indicator of bacterial activity. that it can survive after the destruction of bacterial cells. So this test can be

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used for heat treatment control. Therefore, properly pasteurized milk should not not contain alkaline phosphatase.

5.4.4. Assay of cellular constituents: ATP-luceferin-luciferase system

5.4.5. Electrochemical techniques

Various chemical characteristics of the environment, linked to microbial activity, can be electrically measured and constitute signals indicating the presence of microorganisms.

5.4.5.1. Potentiometry

The decrease in the oxidation-reduction potential correlative to microbial development, and of which we have seen that it could be appreciated by spectroscopy, and can also be appreciated by measurement potentiometric of hydrogen release or oxygen absorption.

5.4.5.2. Ph

By direct measurement with a pH meter or by titrimetry to determine total acidity expressed in quantity of acid. This is how the acidity of milk is considered an index of its bacteriological quality.

5.4.5.3. The concentration of certain ions

Certain ions, released into the environment by microorganisms, can constitute indicators of the nature and extent of contamination, and even the duration of conservation. Among them are the variation of the concentration of lactate and ammonium ions
in dairy.

5.4.5.4. Amperometry

This process is based on the fact that microorganisms themselves are particles negatively charged and that if we immerse in a suspension an electrode made up of a platinum anode and a silver peroxide cathode, microorganisms lose their electrons in contact with the platinum anode and regenerate their charge from the components of the environment. This process generates a current that is measured.

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5.4.6. Other processes

5.4.6.1. Chromatography

Gas chromatography (GC) seems the most suitable but less used. Its importance lies in the fact that it can analyze several substances at once and by the It can then characterize the microorganism by a spectrum of fatty acids that it rejects example. Which allows both to detect the microbial presence and to approach it identification. And this by analyzing the products of its metabolism such as alcohols, fatty acids etc.

5.4.6. 2. Microcalorimetry

The principle is based on the fact that microbial activity releases heat energy, by if a contaminated food product or nutrient medium is introduced into a very sensitive calorimeter, and we compare the thermogenesis to that observed in a control sterile, it is possible to detect the presence of small numbers of microorganisms.

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6. Bacterial identification

6.1. Cultural, morphological and structural characteristics

6.1.1. Cultural characteristics

➢ **Macroscopic examination of cultural characteristics**

The appearance of colonies depends on the environment, duration and temperature of incubation. It can only be described properly from well-isolated colonies. The description of colonies must mention several elements:

- The size
- Shape: rounded, flat, umbilicated, with raised center.
- Surface appearance: smooth, rough.
- Opacity: opaque, translucent, transparent.
- Consistency: oily, creamy, dry, mucous.
- Pigmentation.

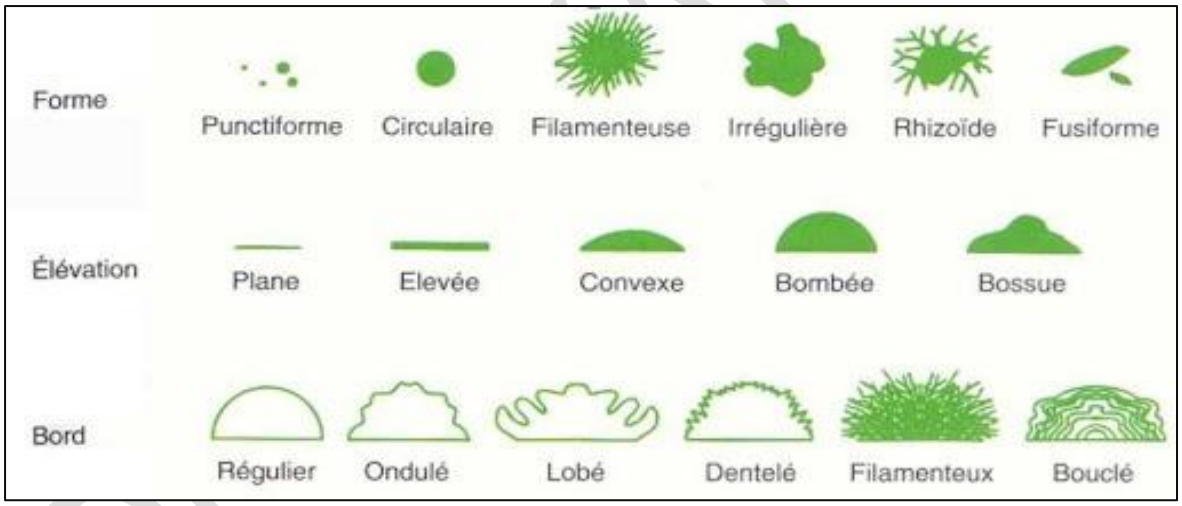

Figure 11:characteristics of colonies

6.1.2. Morphological characteristics

➢ **Microscopic examination after Gram staining**

Microscopic examination after Gram staining initially requires preparation of a smear. A well-isolated colony from a solid medium culture will be taken and suspended in a drop of sterile distilled water. Observation is done using a x100 objective.

This coloring makes it possible to differentiate bacteria according to two criteria:

- their form (bacillus, cocci, etc.),

- their affinity for dyes, in Gram positive and Gram negative.

It takes place in several successive stages and consists of:

1- Fix a smear.
2- Cover the smear with the crystal violet solution. Leave for 1 minute.
3- Pour in the dye. Wash with water.
4- Cover the Lugol preparation. Leave to act for 1 minute.
5- Pour in the Lugol. Wash with water.
6- Discolor with 95° alcohol.
7- Rinse under running water.
8- Cover the slide with the diluted Fuchsin solution. Leave to act for a few seconds and discard the Fuchsin.
9- Wash thoroughly with drained water and dry between two sheets of very clean blotting paper.

❖ **Results**

After this treatment, Gram-positive bacteria are stained well in purple, and Gram-negative bacteria are stained in pink.

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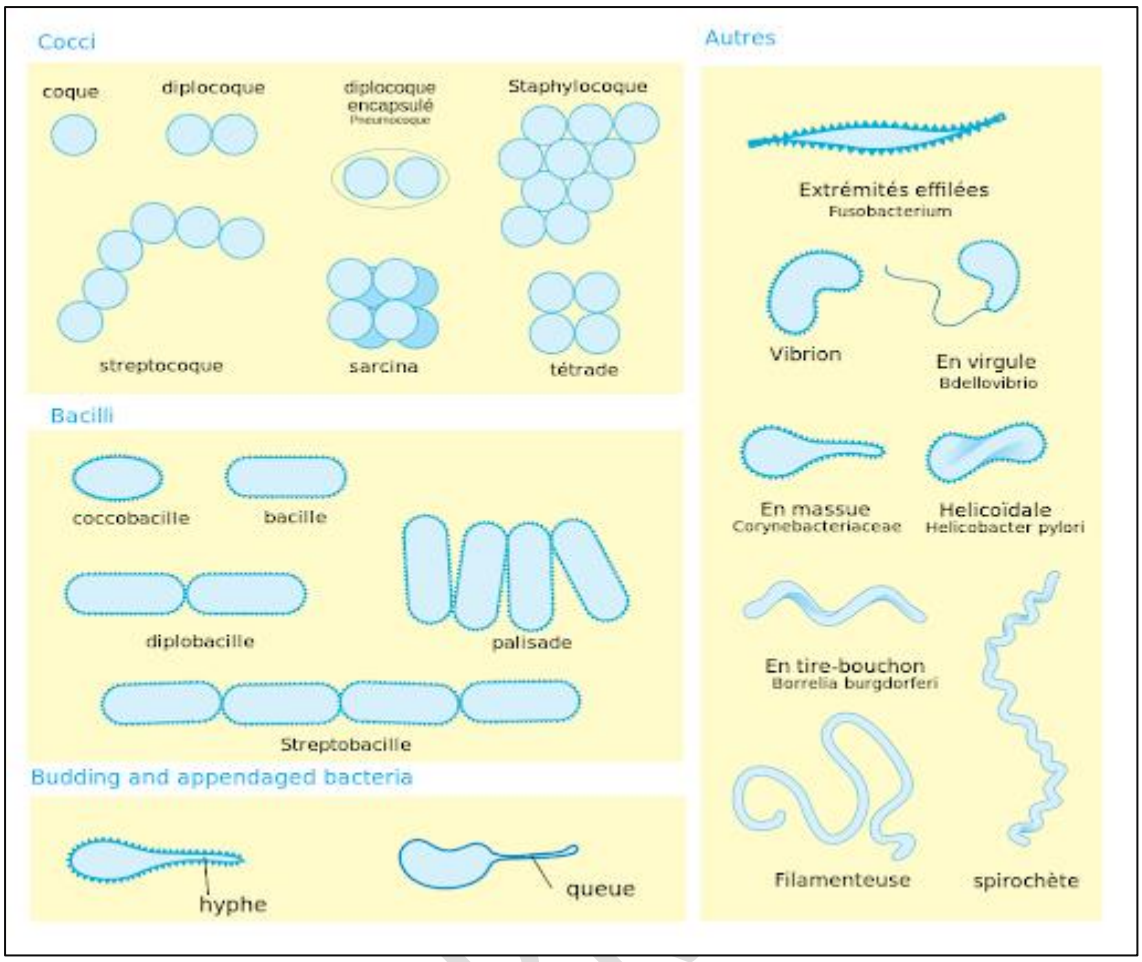

Figure 12: The different bacterial cell forms

6.2. Physiological and biochemical characteristics

6.2.1. Respiratory type

The study of the respiratory type of a microorganism allows to define its relationships with oxygen (some bacteria require O2, others the absence of O2). The medium used is meat-liver agar, conditioned in the Prévot tube, a partial pressure gradient in oxygen is created. This agar is free of nitrates which prevents the development of strict aerobic bacteria having a nitrate reductase, in anaerobic conditions.

6.2.1.1. Operating mode

- Regenerate the medium for 20 min in a bain-marie (cap slightly unscrewed).
- Allow to cool to 45°C (supercooled medium).
- Carry out a spiral seeding in the mass, using a Pasteur pipette loaded with the bacterial suspension to be studied.

- Leave to solidify then incubate for 24 hours at an optimal temperature with the cap unscrewed.

The respiratory type can therefore be observed through the incubated tube which would have allowed the growth of bacteria at different locations in the tube according to the figure below.

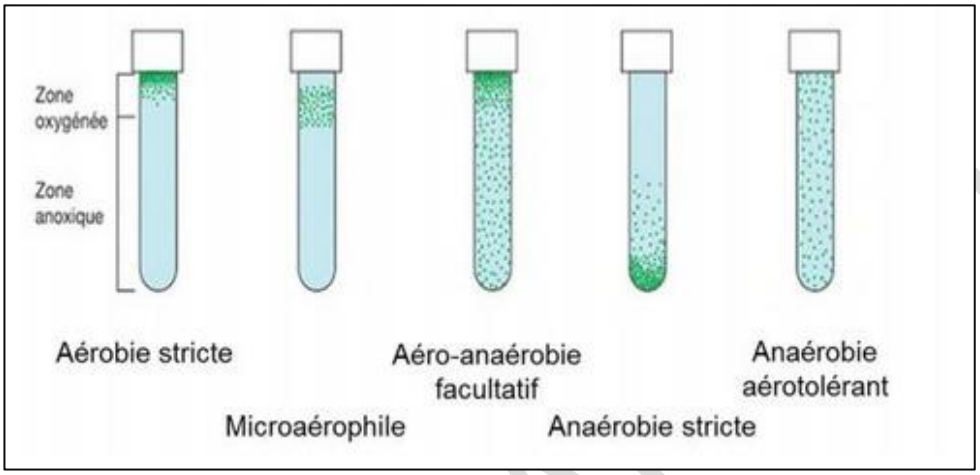

Figure 13: Microbial growth according to oxygen requirement

6.2.2. Mannitol-Mobility

This medium allows the study of mannitol fermentation (biochemical character) and the mobility of the strain (morphological character). This medium can only be used for fermentative bacteria. The presence of a low content of agar (soft agar) makes it possible to move mobile bacteria around the central puncture (the medium is inoculated using a platinum wire or a closed-end Pasteur pipette and incubated at 37°C for 18-24 hours). Reading the use of mannitol is possible thanks to the presence of a pH indicator, phenol red. The use of mannitol acidifies the medium which can thus be revealed by the change of the pH indicator to its acidic color (yellow).

6.2.3. Growth temperatures, halophilicity-osmophilicity and pH:

The assessment of growth ability under hostile conditions is sometimes of great interest in identification. The ability to grow at different temperatures, at different NaCl or sucrose concentrations and at different pH values is carried out on liquid or solid media.

6.2.4. Resistance to antibiotics and inhibitors:

The ability to grow in the presence of certain antibiotics or inhibitory agents is widely used in taxonomy. The presence or absence of multiplication indicates the sensitivity or resistance of the bacteria. This test is performed on liquid or solid media. It is important to emphasize that, unlike Gram-, Gram+ are sensitive with exception (Enterococcus, Lactobacilli, Leuconostoc and Pediococcus spp.) to vancomycin. On the other hand, Gram- are sensitive to colistin and polymyxin, while Gram+ are not.

6.2.5. Enzymatic characteristics

- **Catalase test**(for staphylococci)

It is an enzyme that breaks down hydrogen peroxide into water and gaseous oxygen. The method consists of taking a colony of the germ to be studied on the end of a closed Pasteur pipette which is then immersed in a milliliter of hydrogen peroxide. The release of gas bubbles indicates the presence of the enzyme (Figure 14).

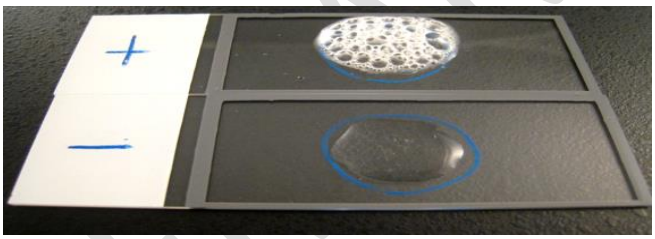

Figure 14: Catalase test (+)

- **Coagulase test**(for staphylococci)

The test demonstrating the ability of bacteria to coagulate plasma is the main test characterizing S. aureus.

The detection test consists of incubating for 4 hours at 37°C a mixture of oxalated plasma from the human and the strain to be tested, preferably from a Chapman agar culture.

The appearance of a clot is observed by tilting the tube at 90°C. The coagulase test, there are very rare strains of S. aureus that do not secrete coagulase. (Figure 15).

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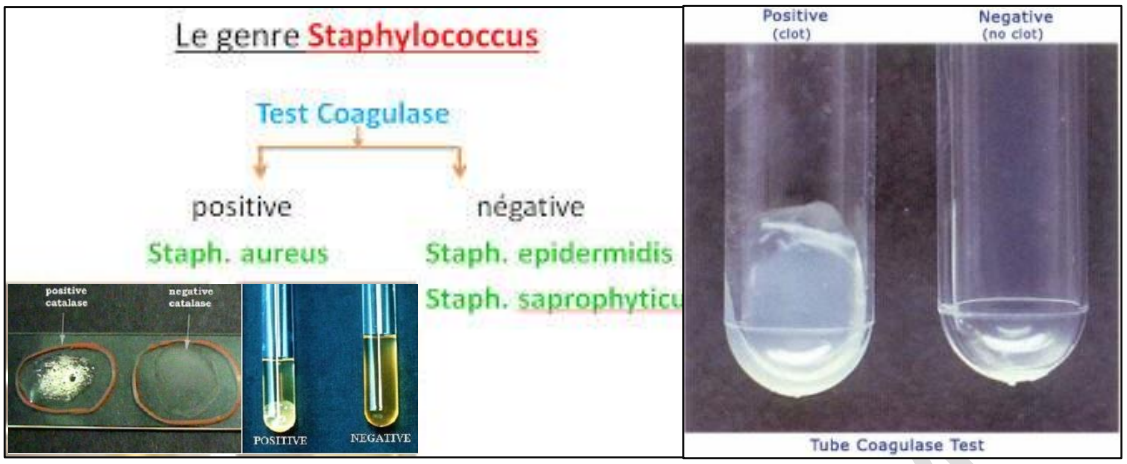

Figure 15: Coagulase test.

- **Oxidase test**

Oxidase testing is performed using ready-to-use commercial disks. Place the disk on a microscope slide, moisten it with two drops of sterile distilled water, and crush the colony tested on the disk. A positive reaction is indicated by a rapid change of the reagent from colorless to violet (Figure 16).

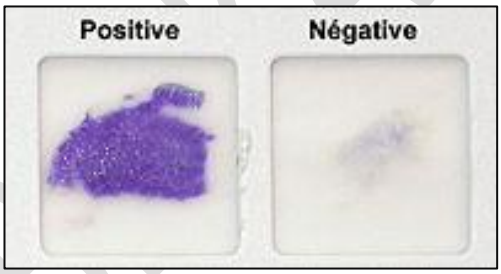

Figure 16: Oxidase test

6.2.6. Biochemical characteristics

6.2.6.1. Miniaturized biochemical test galleries (Api gallery)

Where conventional techniques required one to three weeks, miniaturized ranges allow results to be obtained within 18 to 72 hours (fast). They also allow the identification of approximately 700 bacteria and yeasts, which practically covers a large part of pathogenic microorganisms and nearly 1000 different biochemical tests in common use. The API (analytical profile index) galleries are also easy to produce and high performance (reliable results).

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They are presented in the form of a series of small tubes, called tubules, each corresponding to a specific biochemical test. Each tubule is opened at its upper end by a cup that can be filled, or not, with liquid in order to place the tube in particular conditions. Each tube contains a defined substrate (ONPG, ADH, GEL...) and with which the microorganisms react differently.

When a bacterial suspension of suitable density is distributed in the different alveoli that make up the microgallery (containing dehydrated substrates), the metabolites produced during the incubation period result in spontaneous color changes or changes revealed by the addition of reagents.

It usually includes 20 biochemical tests.

The first gallery that was used is the Api 20 E gallery (Figure 17), intended for the identification of enterobacteria. Currently in the medical bacteriology laboratories galleries Api 20 E, Api 20 NE, Api Staph and Api 20 Strep are also used.

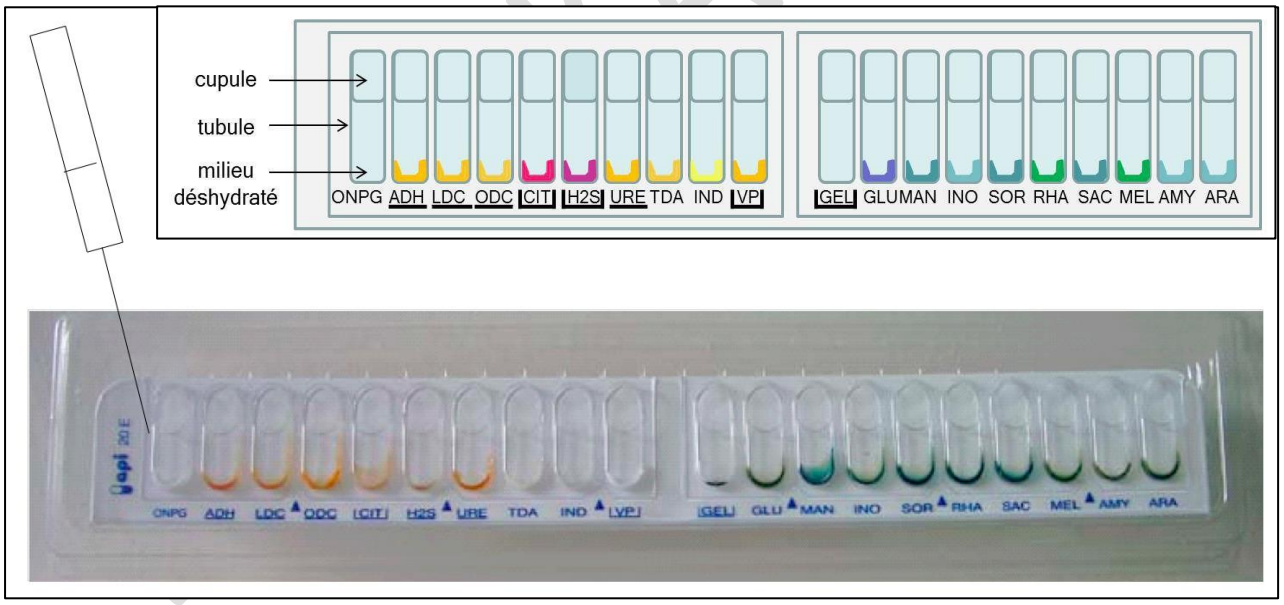

Figure 17: Api Gallery 20 E.

6.2.6.2 Gallery selection

The choice of the gallery to be seeded depends on the results of the study of the morphological, cultural and biochemical characteristics which have been studied previously (oxidase, catalase, etc.) and which are essential for the interpretation of the Api gallery.

-The Api 20 NE gallery is intended for the identification of Gram-negative, undemanding (bacilli other than enterobacteria) and oxidase-positive cocci or bacilli (Pseudomonas and related species, Vibrionaceae, Aeromonadaceae).

-The Api Staph gallery allows the identification of staphylococci and micrococci.

-The Api 20 Strep gallery ensures the identification of streptococci, enterococci and related bacteria (notably some species of the genus Listeria) in 4 or 24 hours.

-For the Api 20 E gallery, enterobacteria (Gram negative, undemanding and oxidase negative bacilli) are easily identified through this gallery. Vibrionaceae, Pseudomonas and Aeromonodaceae are also possible to identify with this gallery.

6.2.6.3. Operating mode

The Api kit seeding operation is carried out according to the following steps:

• Combine the base and lid of an incubation box and distribute approximately 5 ml of distilled water into the cells to create a humid atmosphere.
• Fill the test tubes and cups: |CIT|, |VP|, |GEL|, with the bacterial suspension.
• Only fill the tubes (not the cups) of the other tests.
• Create anaerobiosis in the tests: ADH, LDC, ODC, URE, H2S by filling their wells with paraffin oil.
• Close the incubation box, code and place at 37°C for 18-24 hours.

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6.2.6.4.Reading

Record all spontaneous reactions on the results sheet. If glucose is positive and/or 3 or more tests are positive: reveal the tests requiring the addition of reagents.

-VP test: add a drop of VP1 and VP2 reagent. Wait at least 10 minutes. A bright pink or red color indicates a positive reaction.

-TDA test: add a drop of TDA reagent. A dark brown color indicates a positive reaction.

-IND test: add a drop of Kowacks reagent. A red ring obtained in 2 minutes indicates a positive reaction.

These reactions are read according to the digital profile using the API 20E analytical catalog.

Microtube	Substrat	Caractère recherché	Lecture directe ou indirecte (Test si nécessaire)	Résultat +	Résultat -
ONPG	Ortho-Nitro-Phényl-Galactoside	β-galactosidase	Lecture directe		
ADH LDC ODH	Arginine Lysine Ornithine	Arginine dihydrolase Lysine décarboxylase Ornithine décarboxylase	Lecture directe		
CIT	Citrate	Utilisation du citrate	Lecture directe		
H_2S	Thiosulfate de sodium	Production d'H2S	Lecture directe		
URE	Urée	Uréase	Lecture directe		
TDA	Tryptophane	Tryptophane désaminase	Lecture indirecte Test : ajouter 1 goutte de Perchlorure de Fer		
IND	Tryptophane	Production d'indole	Lecture indirecte Test : ajouter 1 goutte de réactif de Kovacs		
VP	Pyruvate de sodium	Production d'acétoïne	Lecture indirecte (Attendre 10 minutes) Test : ajouter 1 goutte de KOH et d'α-napthol		
GEL	Gélatine emprisonnant des particules de charbon	Gélatinase	Lecture directe		
GLU à ARA	Substrat carboné	Utilisation de substrat carboné	Lecture directe		
NO_2^- / N_2	Nitrates (NO3)	Nitrate réductase	Lecture indirecte dans la cupule GLU Test : ajouter 1 goutte de réactif de Griess Ajouter de la poudre zinc en cas de résultat négatif		

Figure 18: Api Gallery Reading Table

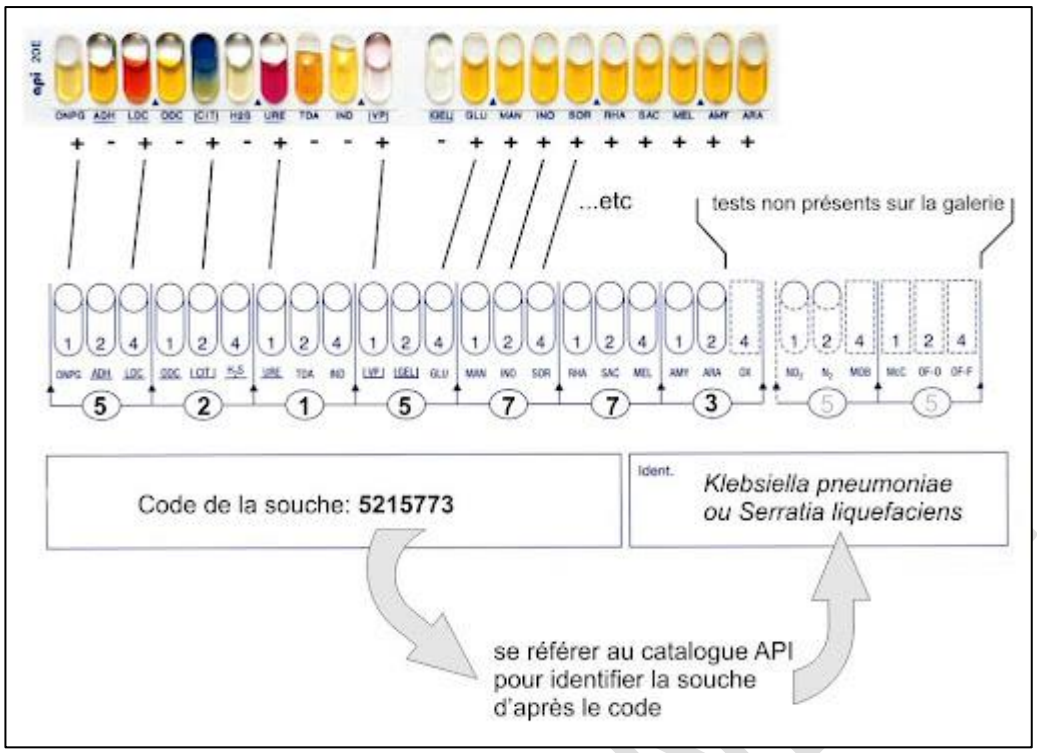

Figure 19: Interpreting the results of the Api gallery

6.3. Immunological characteristics

Serological reactionsbacteria-antibody type (agglutination reaction) or antigen-antibody type (precipitation reaction) are used in taxonomy mainly for Enterobacteria which contain three types of antigens: H, O, and K; and streptococci of which the most important is type C: A, B, C, D, N. These serological tests are mainly carried out using the Lancefield technique based on the use of polysaccharides (notably polysaccharide C) from the cell envelope as an antigen.

6.4. Pathogenic power

Coagulase: this character alone allows to affirm the presence of St. aureus which is coagulase+ from other Staphylococcus which are coagulase- (St. epidermidis, St. saprophyticus). St. aureus produces two types of coagulase: (1) free coagulase (extracellular enzyme) and (2) bound coagulase (protein associated with the wall). Both enzymes are capable in vitro of coagulating rabbit plasma (the formation of an insoluble fibrin clot).

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6.5. Genomic identification

Since enormous progress has been made in molecular genetics, bacteriology has a new means of identifying a strain: the polymerase chain reaction or PCR method.
This method is based on the search and multiplication of a specific fragment of DNA from the bacteria.
However, it is reserved for only a few strains because it represents a significant investment in equipment and reagents.

The Principle

A PCR identification takes place in 3 parts.

The first step is to synthesize DNA fragments with sequences identical to those flanking the desired sequence (= the specific part). This can be done using an automatic synthesizer and the fragments obtained, called "primers", serve as a primer for DNA synthesis. They are generally about 20 nucleotides long.

The second step is the PCR reaction itself. It consists of three steps, repeated many times, in order to obtain approximately 1 million copies of the specific fragment.

- **Denaturation**bacterial DNA by heat. The two strands of DNA separate
- **Hybridization**DNA strands with the primers. In fact, care was taken to add these in excess so that the probability of DNA/primer hybridization is greater than the probability of DNA/DNA rematching
- **Elongation**new DNA strands using triphosphate nucleotides and a heat-insensitive DNA polymerase (Taq polymerase, an enzyme extracted from an archaeobacterium living in hot springs: Thermophilus aquaticus).

This cycle, usually carried out in a special device, the thermocycler, is generally repeated around thirty times.
The last step is the revelation. In most cases, we use the property of ethidium bromide to bind permanently to DNA and to be fluorescent under UV. Since the sequence, and therefore the size, of the specific fragment is known, it is enough to check that we have a "band" at the desired size to conclude.
Hemolysins α, ß and γ: enzymes responsible for the lysis of red blood cells, they are demonstrated by culture on blood agar. α-haemolysins (greenish zone due to metmyoglobin,

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example: S. pneumonia); ß-haemolysins (clear halo due to the release of haemoglobin, example: St. aureus); γ-haemolysins (no modification, no haemolysis around the colonies, example: E. faecalis).

DNAse: enzyme that destroys the nucleus of cells, it is highlighted on media containing DNA. DNA hydrolysis is characterized by a clear zone, example: St. aureus+ and St. epidermidis-

7. Carrying out the check

7.1 Control of raw materials

Microbiological control of raw materials must make it possible to verify that they do not contain microorganisms that could interfere with the manufacturing process, nor microorganisms that could alter the product. Here, a distinction must be made between:

Fermentation industries, where the control is most often a sterility control of the environment / a microbiological cleanliness control of the leaven; Other industries, where the control consists of looking for potentially dangerous microorganisms (aerobic germs, yeasts and molds, Clostridium spp., Salmonella spp., Staphylococcus aureus, Escherichia coli, Coliforms, Fecal coliforms, Fecal streptococci, etc.), and analyzing the usual physicochemical parameters (H%, MS%, pH and acidity, etc.).

7.2. Control of sourdoughs

The control of sourdoughs must allow the detection of contaminants present at levels that are often very low compared to culture cells. Three main types of sourdoughs are used in the fermentation industries and the search for contaminants is done by culture techniques or by microscopic techniques.

(1) Saccharomyces sourdoughs: the most frequent contaminants are wild yeasts, lactic and acetic bacteria;

(2) Yeast and mold starters: the most common contaminants are bacteria;

(3) Bacterial starters: contaminants are frequently other bacteria and bacteriophages.

7.3. Manufacturing control

Manufacturing control consists of monitoring the manufacturing process:

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Fermentation industries, where control essentially consists of monitoring both the development of the leaven and the appearance and development of contaminants (the most widely used microscopic techniques: counting technique and coloring technique);
Other industries, where control essentially consists of monitoring processing parameters, but also of searching for and counting potentially dangerous microorganisms (aerobic germs, yeasts and molds, Clostridium spp., Salmonella spp., Staphylococcus aureus, Escherichia coli, Coliforms, Fecal Coliforms, Fecal Streptococci, etc.).

7.4. Cleaning and disinfection control

Cleaning and disinfection are generally carried out either systematically or between two successive manufacturing operations. In both cases, the control is to verify the effectiveness of the cleaning and disinfection operation in destroying unwanted microorganisms. For this, different techniques are used: contact boxes and slides for the microbiological control of surfaces, swabbing for the microbiological control of less accessible devices (valves, taps, cannulas, etc.).
Ambient air is also subject to microbiological control, simply by using Petri dishes filled with culture medium, which are left open for a maximum of 15 minutes.

7.5. Control of finished products

Microbiological control of finished products concerns their hygienic quality and their marketable quality. This control consists of the search for and counting of potentially dangerous microorganisms (aerobic germs, yeasts and molds, Clostridium spp., Salmonella spp., Staphylococcus aureus, Escherichia coli, Coliforms, Fecal coliforms, Fecal streptococci, etc.) and concludes that the product complies with the standards (microbiological criteria or specifications).

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