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التعليم العالي و البحث العلمي

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Forword

This course material is tailored to the undergraduate programs in Biological Sciences and is intended for second-year (L2) students. The practical work (PW) provides a foundation in general microbiology, training students in this field and adapting it to practical work in microbiology laboratories.

The practical works correspond to the expected handout teaching skills, and this handout is the microbiology practical knowledge result and students adaptation for specializations that include microbiology instruction. It is therefore an effective teaching tool using a relatively simple, clear, and well-organized style.

The manuscript is divided into four main chapters arranged in chronological order of microbiological analyses. The first chapter concerns the basic rules and safety instructions to be followed in a microbiology laboratory, personal protective equipment (PPE), asepsis concepts and recognized sterilization methods in microbiology. Basic knowledge for proper handling. In the second chapter, culture media, inoculation techniques applied in microbiological analysis and bacterial isolation techniques are explained because they constitute a crucial step to begin a microbiological analysis of any sample, whether in the medical field (biological samples, examples: urine, fecal matter, pus, blood, etc.) or in the agri-food field (microbiological food quality control). This chapter presents the different procedures of isolation, inoculation, observation and morphological characterization of bacterial colonies by macroscopic examination as an initial step in bacterial identification, the main objective of practical work in microbiology (knowing how to identify bacteria).

The other chapters present the other procedures to be followed in bacterial identification, moving from macroscopic examination to microscopic bacteria examination (wet state and Gram staining), and subsequently to a series of biochemical and enzymatic tests performance and an antibiogram to ultimately determine the antibiotic resistance profile of the identified bacterium.

The handout objective is to present in a concise and precise manner the basic data of a microbiological analysis.

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I sincerely thank the professors involved in the manuscript evaluation.

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µm : Micrometer

ADH : Arginine dehydrolase

BHIB : Heart-brain broth

Ca^{2+} : Calcium

CASFM : French Society for Microbiology - Antibiotic Susceptibility Testing Committee

Cl^- : Chlorine

CLP : Classification Labelling Packaging

Cu : Copper

EMB : Methylene blue eosin agar

Fe : Iron

FMS-AC/EUCAST : European Committee on Antimicrobial Susceptibility Testing

GN : Nutrient agar

H_2S : Hydrogen sulfide

K^+ : Potassium

LDC : Lysine decarboxylase

SMCMP : Study media for the carbohydrate metabolism pathway.

Mg^{2+} : Magnesium

N_2 : Nitrogen

Na^+ : Sodium

NaCl : Sodium chloride

NO_2^- : Nitrites

NO_3^- : Nitrates

ODC : Ornithine decarboxylase

ONPG : Ortho-Nitrophenyl-galactoside

pH : Hydrogen potential

PO_4^{-2}: Phosphate ion

SGH : Globally Harmonized System

SO_4^{2-} : Ions suffer

SS : Salmonella-Shigella Agar

TSI : Triple sugar iron

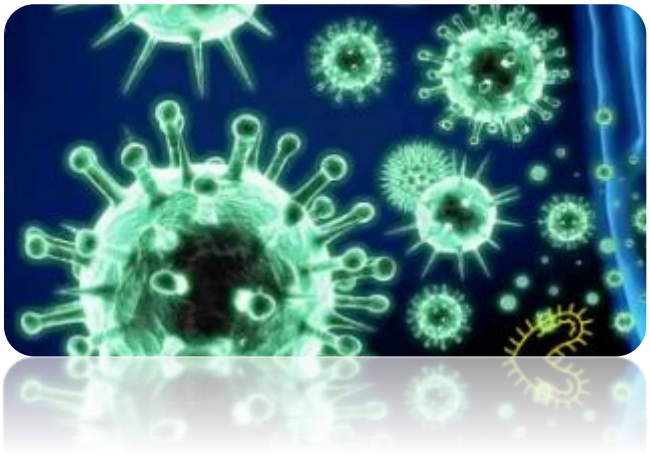
UV : Ultraviolet

VF : Meat-liver

VIH : Immune deficiency virus

Zn : Zinc

General introduction



Introduction

Microbiology is the scientific discipline concerned with the study of microorganisms, including their morphology, physiology, genetics, ecology, and interactions with other living organisms and the environment. It covers both cellular microorganisms (bacteria, archaea, fungi, protozoa, and microscopic algae) and acellular infectious agents such as viruses and prions (Libre Texts, 2024; National Library of Medicine, 2019). Microbiology is divided into several branches, depending on the studied type of "microbe": bacteriology, virology, mycology, parasitology, and phycology (unicellular algae) (Leclerc, 1995). In this module, we will study bacteriology, the isolation and identification of bacteria, as an introduction to microbiology.

Within the same microbial family, we can distinguish between beneficial microbes belonging to our common resident or native flora and pathogenic microbes of the transient or allochthonous flora. Through this module, we will investigate more details concerning the different bacteria of the microbial world, mentioning specific examples for a better field knowledge acquisition. Students will learn the various basic rules for working safely in a microbiology laboratory, regarding that it will be their first training in this field, so they must be more careful when handling microorganisms, whether beneficial or pathogenic, and the personal protective equipment (PPE) that students must wear before handling in a microbiology laboratory.

Furthermore, the teacher will provide more details on this module and more specifically the main steps to follow during a microbiological analysis, whether in the medical analyses field or in the food microbiological quality control, the student will therefore be trained on both axes, the medical and the agri-food axis. Through this module, students will become competent in the bacteriological analyses field, starting with bacteria isolation, progressing to bacterial identification and antibiograms achieving.

PW 1 : Basic rules for working safely in a microbiology laboratory



I. Introduction

A microbiology laboratory is a specialized facility where microorganisms are isolated, cultured, identified, and analyzed using microbiological, biochemical, immunological, and molecular techniques. It plays a critical role in the diagnosis of infectious diseases, antimicrobial susceptibility testing, epidemiological surveillance, research, and the monitoring of microbial contamination in clinical, environmental, food, and pharmaceutical samples (European Centre for Disease Prevention and Control (ECDC), (2024).

When working in a microbiology laboratory, students must strictly adhere to all biosafety regulations and laboratory safety guidelines. Compliance with these rules is essential not only to ensure their own safety but also to protect their colleagues and the surrounding environment, as pathogenic microorganisms may be transmitted through accidental exposure. Consequently, all microbiological work must be conducted according to established biosafety practices, regardless of whether the microorganisms handled are pathogenic or non-pathogenic (World Health Organization, 2024).

Laboratory personnel who manipulate infectious agents are at risk of acquiring laboratory-associated infections (LAIs) through accidents or unrecognized exposure incidents. The level of risk depends on several factors, including the virulence and pathogenicity of the microorganism, the infectious dose, the route of exposure, and the physiological and immunological status of the exposed individual. Laboratory-acquired infections may occur through accidental ingestion, inhalation of infectious aerosols, inoculation via contaminated sharp instruments, or contact of infectious material with broken skin or mucous membranes. Among these routes, accidental ingestion represents one of the most common causes of laboratory-acquired infections, particularly when handling enteric pathogens such as *Salmonella* spp., *Shigella* spp., and *Escherichia coli* O157:H7.

To minimize these risks, Biosafety Level 2 (BSL-2) practices must be implemented whenever handling microorganisms that present a moderate risk to laboratory personnel and the environment. These practices include:

- Providing personnel and students with appropriate training in the safe handling of pathogenic microorganisms under the supervision of qualified instructors or researchers.
- Restricting laboratory access while microbiological procedures are being performed.

- Exercising extreme caution when handling contaminated needles, scalpels, and other sharp instruments.
- Performing procedures that may generate infectious aerosols or splashes inside a certified biological safety cabinet (BSC) whenever possible.
- Wearing appropriate personal protective equipment (PPE), including laboratory coats, gloves, and eye protection, and maintaining strict aseptic techniques throughout all laboratory procedures (American Society for Microbiology, 2023).

Students should also be aware that hazards in the microbiology laboratory are not limited to biological agents. Chemical hazards arise from the use of disinfectants, stains, acids, solvents, and other laboratory reagents, while physical hazards are associated with laboratory equipment such as autoclaves, centrifuges, incubators, Bunsen burners, and electrical devices. Consequently, all equipment should be operated only according to established laboratory procedures and under appropriate supervision when required. Strict compliance with microbiological safety procedures, chemical safety regulations, and good laboratory practices (GLPs) is fundamental for preventing laboratory accidents, minimizing exposure risks, and ensuring a safe and effective learning environment.

II. Objectives

- To understand the various safety instructions and standard practices in a microbiology laboratory.
- To understand the different instruments, materials, and laboratory equipment.

Part I

III. Standard Safety Practices in Microbiology

The safety guidelines given below apply to all microbiology laboratories regardless the biosafety level (World Health Organization, 2024; American Society for Microbiology, 2023; International Organization for Standardization, 2020).

III.1. Limit access to the laboratory

- Place pictograms or warning pictograms indicating a biological hazard on all microbiological laboratory doors and on all equipment such as incubators, fume hoods, refrigerators, freezers, and autoclaves used for laboratory work.

- Do not allow children under 12 years of age or animals into the laboratories.



Figure 1: Biological hasard pictogram. Source: Adapted from ISO 7010:2019.

III.2. Lab Coat (Personal Protective Equipment: PPE)

- Wearing long-sleeved, button-down cotton lab coats is mandatory while working in the microbiology laboratory.
- Lab coats must be removed upon leaving the laboratory and left it in.
- All protective clothing is either destroyed in the laboratory or cleaned by the institution. Laboratory staff must never take them home.

III.3. Handwashing

- Each laboratory must be equipped with a handwashing sink.
- Frequent handwashing is one of the most effective procedures for preventing infections in the laboratory.
- Handwashing is also necessary before beginning any procedures to prevent contamination of the sterile products to be used.
- Hands must be washed with antibacterial soap before leaving the laboratory or after touching infectious materials.

III.4. Protective Gloves (PPE)

- Regardless the infectious material type, wearing gloves is mandatory before any lab. work, as well as when performing hazardous procedures when there is a risk of skin contamination or when the students have cuts or other injuries on their hands.

- Wearing gloves therefore protects the student against contracting pathogenic germs and their transmission through the skin or skin lesions.
- Gloves must always be worn when handling clinical samples, biological fluids, contaminated food, and human or animal tissues.
- Students should assume they may be infected with hepatitis B virus, HIV, other bloodborne pathogens, or *Mycobacterium tuberculosis*.
- Students should remove their gloves when they are contaminated or when finishing working with infectious materials.
- Gloves should not be worn outside the laboratory.
- Do not use the telephone or open doors while wearing gloves that have already been used during laboratory procedures.

Note: Samples to be handled should always be considered potentially positive for hepatitis B virus, HIV, other bloodborne pathogens, or *Mycobacterium tuberculosis*. These samples should be handled in fume hoods with appropriate protective equipment such as goggles, gloves, a mask, or other means of face protecting from splashes or aerosols.

- Discard all used gloves with the rest of the single-use equipment intended for autoclaving.
- Wash hands immediately after removing gloves.

III.5. Other

- Talking is prohibited during procedures to avoid contaminating the sterile work area (see chapter below).
- Masks must be worn during procedures to protect the face from splashes (drops of liquid that have been sprayed and can reach the mouth and nose) or aerosols.
- Students must avoid sudden movements during procedures to prevent workplace accidents.
- Running is prohibited inside the laboratory to prevent workplace accidents.

- Wearing jewelry and accessories (rings, watches, bracelets, nose piercings, etc.) is prohibited in the laboratory. These items must be removed before entering the laboratory as they can be a significant means of transmitting microorganisms.
- Wearing false nails, false eyelashes, and contact lenses is prohibited in a microbiology laboratory (risk of contamination and germs transmission, workplace accidents risk).
- Hair must be tied back to avoid hindering students during experiments and to prevent hair from coming into contact with the Bunsen burner blue flame (workplace accidents).
- Hats and caps are not permitted in the laboratory.
- High heels are not permitted in the laboratory.
- Eating, drinking, and smoking are prohibited in the work areas. Food must be stored outside the laboratory in designated storage areas.
- Do not leave personal items such as handbags, mobile phones, or glasses on the manipulation surfaces.
- Oral sampling with a pipette: All oral pipetting is strictly prohibited. Use a bulb syringe or a pipette filler.

✓ ***Sharps***

- Use extreme caution with contaminated sharps, including needles and syringes, blades, pipettes, capillary tubes, and scalpels.
- Dispose of sharps in designated sharps containers.
- To avoid finger pricks, do not attempt to bend or break needles or recap them, as this can lead to finger pricks.
- Sharp objects that are not discarded must be placed in a clearly labeled container for decontamination before cleaning.
- Do not touch broken glass directly with your hand; remove it using mechanical means such as brushes or tongs.

- ✓ ***Splashes and Aerosols***

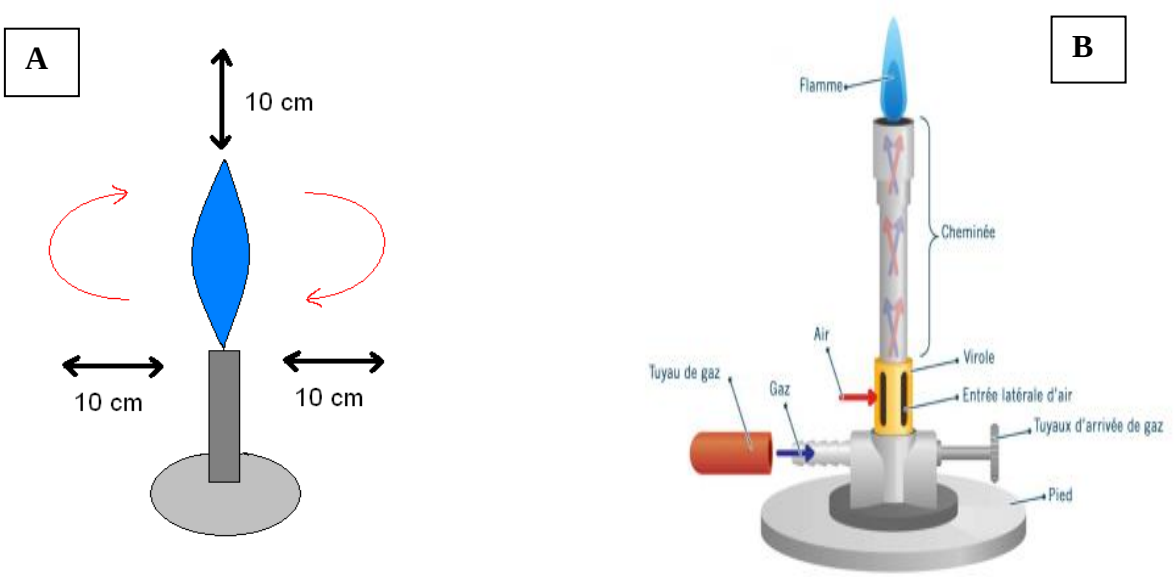
- Carefully perform all procedures to avoid splashing or aerosol formation.
- Avoid techniques that tend to produce aerosols.
- Cool inoculation platinum loops and inoculating wires by holding them in the air for 5 to 10 seconds before touching colonies or clinical material.
- Dry inoculation platinum loops containing infectious material with hot air over a Bunsen burner before sterilizing them (to avoid splashing and aerosol formation).
- Perform centrifugation or vortexing in closed containers.
- Never cut needles or remove them from syringes before autoclaving.
- Centrifuge biological fluids only in containers with child-resistant caps.

IV. Workbenches disinfection

Disinfecting workbenches is a crucial step before any manipulation in the microbiology laboratory. The entire workbench is disinfected using a powerful disinfectant such as sodium hypochlorite (bleach), which destroys all microorganisms present on the surface.

V. Sterile manipulation zone creation

Manuals in a microbiology laboratory can only be performed after a sterile work area has been established. To do this, the student needs a specific instrument: a Bunsen burner. When lit, this burner generates a flame that the student handles with care. Students use the blue flame to create the sterile work area, not the red one. The air around this flame is 99% sterile because the heat generated by the flame destroys all microorganisms (American Society for Microbiology, 2023).



Sterile work area

Bunsen burner

Figure 2: A: Creation of the sterile zone using the Bunsen burner, B: Components of the Bunsen burner.

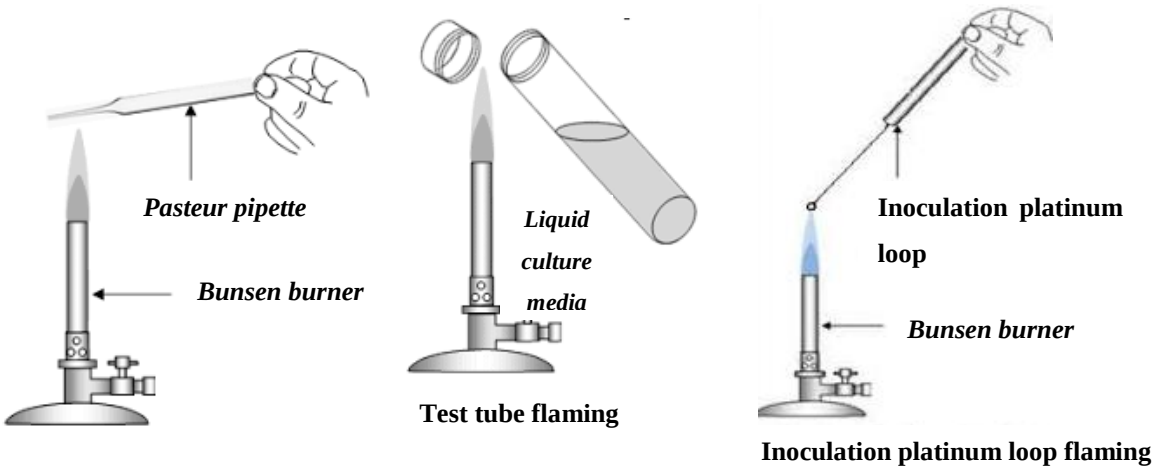


Figure 3: Sterilization of the test tubes and inoculation platinum loop using the Bunsen burner flame.

VI. Asepsis concepts

Aseptic technique consists of preventing the sterile work area or the sterile zone contamination by foreign microorganisms (bacteria, parasites, etc.). It also involves the prior sterilization of all equipment and glassware to be used and avoiding contact between these materials and non-sterile equipment to prevent contamination (Centers for Disease Control and Prevention, & National Institutes of Health, 2020).

VII. Microbiology laboratory sterilization equipment

The figure in below represent the different equipment's used for sterilization in microbiology laboratory.



Autoclave



Pasteur oven



Watter bath



UV-Hood

Figure 4: Sterilization equipment.

A



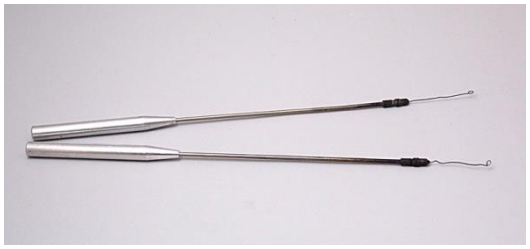
Bunsen burner



Pasteur pipette



Pasteur pipette



Inoculation platinum loop



Test tube



Test tubes rack



Test tube



Beaker



Funnel



Magnetic bar



Magnetic stirrer



Electronic scale

B



Swab



Petri dishes



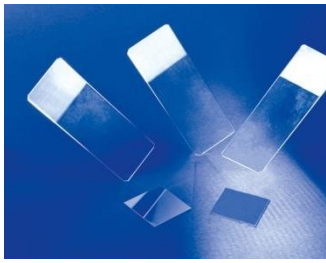
Micropipette



Tip



Glass bottle



Blade and slats



Microscope



Incubator



Vortex



Scalpel



Scalpel



Pliers

Figure 5: Laboratory instruments and glassware (A-B).

Part II

VIII. Chemical products danger


Etiquette apposée sur un flacon de toluène (selon le règlement CLP)

The acronym "CLP" stands for "Classification, Labelling, Packaging".

A hazard class is a category used to classify chemicals or substances according to the type of hazard they present. Under the Globally Harmonized System of Classification and Labelling of Chemicals (GHS), hazards are grouped into three main categories: physical hazards, health hazards, and environmental hazards. Physical hazards include properties such as flammability, explosiveness, oxidizing capacity, and corrosiveness to metals. Health hazards encompass effects such as acute toxicity, skin and eye irritation, carcinogenicity, mutagenicity, reproductive toxicity, and specific target organ toxicity. Environmental hazards refer primarily to substances that are hazardous to the aquatic environment or that may cause long-term adverse effects on ecosystems. Hazard classification enables the appropriate labeling, handling, storage, and safe use of chemicals in the laboratory (Occupational Safety and Health Administration, 2024; United Nations, 2023).

The CLP regulation defines 28 hazard classes:

- 16 physical hazard classes.
- 10 health hazard classes.
- 1 environmental hazard class covering hazards to the aquatic environment.

VIII.1. Physical hazard classes

- Explosives
- Flammable gases
- Aerosols
- Oxidizing gases
- Gases under pressure
- Flammable liquids
- Flammable solids
- Self-reactive substances and mixtures
- Pyrophoric liquids
- Pyrophoric solids



- Self-heating substances and mixtures
- Substances and mixtures which, in contact with water, release flammable gases
- Oxidizing liquids.
- Oxidizing solids.
- Organic peroxides.
- Substances or mixtures corrosive to metals.

VIII.2. Health hazard classes



- Acute toxicity.
- Skin corrosion/skin irritation.
- Severe eye damage/eye irritation.
- Respiratory or skin sensitization.
- Germ cell mutagenicity.
- Carcinogenicity.
- Reproductive toxicity.
- Specific target organ toxicity – single exposure.
- Specific target organ toxicity – repeated exposure.
- Aspiration hazard.

VIII.3. Environmental hazard classes



- Hazards to the aquatic environment.
- Hazards to the ozone layer.

The hazard pictograms prescribed by the CLP Regulation are derived from the GHS and are of 9 pictograms. They consist of "a black symbol on a white background within a red border thick enough to be clearly visible." Each pictogram has a code composed as follows: "GHS" + "0" + 1 number (European Parliament, & Council of the European Union, 2008).

Labeling according to the CLP Regulation:

The information required for CLP labeling is as follows:

- Supplier identity
- Product identifiers
- Hazard pictograms
- Signal word

- Hazard statements
- Precautionary statements
- Additional information section
- Nominal quantity for products made available to the general public (unless this quantity is specified elsewhere on the packaging).



Figure 6: Globally Harmonised System of chemicals classification and labelling.

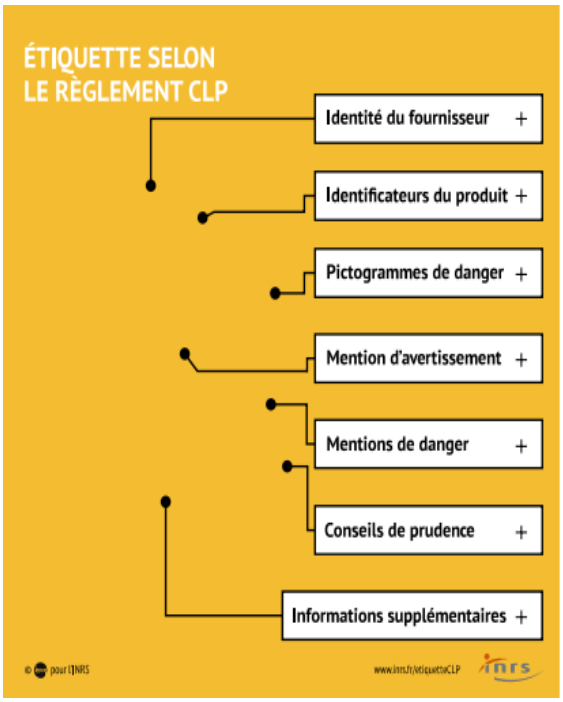


Figure 7: Labeling according to the CLP regulation.



Figure 8: Labeling according to the CLP regulation.

Part III

IX. Sterilization and disinfection techniques used in the microbiology laboratory

Sterilization is an essential procedure in microbiology that aims to completely eliminate or destroy all forms of microbial life, including vegetative microorganisms, bacterial spores, fungi, and viruses, from materials, equipment, culture media, and other laboratory items. It is a mandatory step during the preparation of microbiological media and laboratory equipment, as well as before cleaning, reuse, or disposal of contaminated materials to prevent the spread of infectious agents (World Health Organization, 2024).

Sterilization is applied to inanimate objects and ensures that an item is free of viable microorganisms. Once sterilized, materials must be handled and stored under appropriate aseptic conditions to maintain their sterility and prevent recontamination before use (Centers for Disease Control and Prevention, & National Institutes of Health, 2020).

The sterilization method selected depends on the nature of the material or product being treated, its heat sensitivity, and its intended use. The most commonly used sterilization methods in microbiology laboratories include moist heat sterilization (autoclaving), dry heat sterilization, filtration, radiation sterilization, and chemical sterilization (International Organization for Standardization, 2018).

IX.1. Dry heat sterilization (microbial proteins oxidation)

IX.1.1. Bunsen burner flames

IX.1.1. Bunsen burner flame sterilization

The Bunsen burner is commonly used in microbiology laboratories to facilitate aseptic techniques. The blue, non-luminous flame generates an upward convection current that helps reduce the deposition of airborne microorganisms onto sterile materials, thereby creating an aseptic working environment. In addition, direct exposure to the flame provides rapid sterilization of heat-resistant laboratory instruments by incinerating microorganisms present on their surfaces (American Society for Microbiology, 2023).

This method is routinely used for the sterilization of inoculating loops and needles, the mouths of culture tubes and flasks before and after opening, and the tips of glass Pasteur

pipettes. Care must be taken to use the blue flame, which burns at a higher temperature and more efficiently than the yellow luminous flame, while observing all laboratory fire safety precautions.

IX.1.2. Hot Air oven (Pasteur oven)

The hot air oven (Pasteur oven) is a dry heat sterilization device used to sterilize heat-resistant materials by exposing them to hot air at controlled temperatures for a specified period. Dry heat destroys microorganisms through oxidative processes and protein denaturation. Because bacterial spores are more resistant to dry heat than to moist heat, higher temperatures and longer exposure times are required (International Organization for Standardization, 2018).

Common sterilization conditions include 160°C for 2 hours, 170°C for 1 hour, or 180°C for 30 minutes, depending on the nature and volume of the materials being sterilized.

The hot air oven is suitable for sterilizing metal instruments, glassware (such as Petri dishes, flasks, pipettes, and test tubes), porcelain items (e.g., mortars and filters), powders, and certain oils. However, it is not suitable for sterilizing culture media, aqueous solutions, rubber, plastics, fabrics, or other heat-sensitive materials, as these may melt, deform, or be damaged during the process.

Before sterilization, glassware should be thoroughly cleaned and completely dry. Items may be wrapped in kraft paper or aluminum foil or plugged with cotton to maintain sterility after processing. Following sterilization, the materials should remain inside the oven until they have cooled completely to prevent thermal shock and contamination. Sterile items should then be stored in a clean, dry, and dust-free environment until use.

IX.2. Moist heat sterilization

Moist heat sterilization is one of the most effective and widely used methods for eliminating microorganisms. It relie on the combined action of saturated steam and high temperature, which rapidly transfers heat to microbial cells. This process causes the denaturation and coagulation of cellular proteins and enzymes, disrupts cell membranes, and damages nucleic acids, leading to the irreversible destruction of microorganisms, including bacterial spores when appropriate temperature and pressure conditions are applied (World Health Organization, 2024; Centers for Disease Control and Prevention, & National Institutes of Health, 2020).

Compared with dry heat, moist heat penetrates materials more efficiently, allowing sterilization to be achieved at lower temperatures and in shorter exposure times. The most common method of moist heat sterilization is autoclaving, which uses saturated steam under pressure, typically at 121°C under 15 psi (103 kPa) for 15–20 minutes, depending on the nature and volume of the material being sterilized.

Moist heat sterilization is suitable for culture media, aqueous solutions, surgical instruments, glassware, laboratory waste, textiles, and other heat- and moisture-resistant materials. However, it is not appropriate for oils, powders, or materials that are damaged by moisture or high temperatures.

IX.2.1. Autoclaving (The most widely used and effective sterilization method)

Autoclaving is the most widely used and effective method of moist heat sterilization in microbiology laboratories. It employs saturated steam under pressure to destroy all forms of microbial life, including vegetative cells, fungi, viruses, and bacterial spores. The combination of steam, pressure, and temperature ensures rapid heat penetration and efficient sterilization.

The operating principle of an autoclave is based on the relationship between pressure and the boiling point of water. Under normal atmospheric pressure (101.3 kPa or 760 mmHg), water boils at 100°C. When pressure is increased inside the sealed chamber, the boiling point of water rises, allowing saturated steam to reach temperatures above 100°C. Standard sterilization conditions are 121°C at 15 psi (103 kPa) for 15–20 minutes, although longer exposure times may be required for large volumes or densely packed materials.

Autoclaving is suitable for sterilizing culture media, aqueous solutions, microbiological waste, glassware, surgical instruments, rubber goods, and many autoclavable plastic materials that are resistant to heat and moisture. However, it is not suitable for oils, powders, volatile substances, or heat-sensitive materials that may be damaged by steam or high temperatures.

Before each sterilization cycle, the water level and proper functioning of the autoclave should be verified. Materials should be arranged to allow free circulation of steam throughout the chamber. Bottles, flasks, and test tubes should not be overfilled and their caps should be loosened or covered with aluminum foil or cotton plugs to prevent pressure buildup while allowing steam penetration. After completion of the sterilization cycle, the pressure must

return to atmospheric pressure before the autoclave is opened, and sterilized materials should be allowed to cool before handling (World Health Organization, 2024).

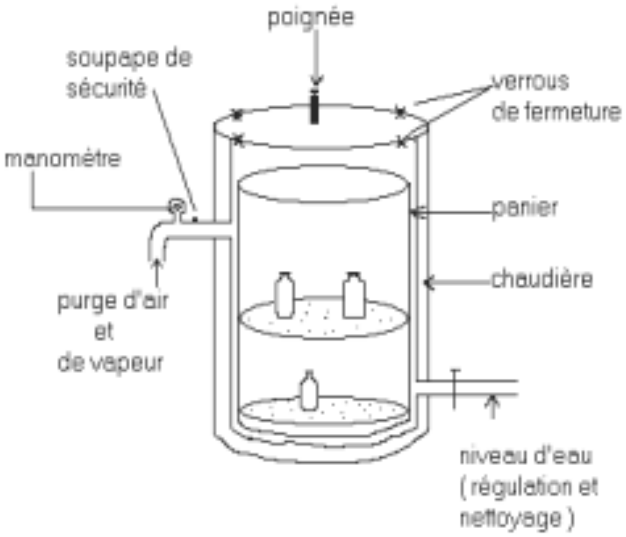


Figure 9: Autoclave (World Health Organization, 2024; Centers for Disease Control and Prevention & National Institutes of Health, 2020).

IX.3. Pasteurization (Not effective against bacterial spores)

Pasteurization is a mild heat treatment used to reduce the number of pathogenic and spoilage microorganisms in foods and beverages without achieving complete sterilization. Unlike sterilization, pasteurization does not destroy bacterial spores, and therefore pasteurized products generally require refrigeration to limit microbial growth after processing (World Health Organization, 2024; Food and Agriculture Organization of the United Nations, & World Health Organization, 2023).

The process consists of heating the product to a specific temperature for a defined period, followed immediately by rapid cooling to inhibit the growth of surviving microorganisms. Heat treatment may be carried out using water baths or industrial heat exchangers, depending on the nature and volume of the product (World Health Organization, 2024; Food and Agriculture Organization of the United Nations, & World Health Organization, 2023).

The principal pasteurization methods include:

- Low-Temperature Long-Time (LTLT) pasteurization: heating at 63°C for 30 minutes.

- High-Temperature Short-Time (HTST) pasteurization: heating at 72°C for 15 seconds.
- Ultra-High Temperature (UHT) treatment: heating at 135–150°C for 2–5 seconds, producing commercially sterile products that can be stored at room temperature until opened.

Pasteurization is widely used for milk, cream, fruit juices, cider, beer, and other beverages to improve microbiological safety while preserving their nutritional and sensory qualities.

IX.4. Tyndallization (Fractional sterilization)

Tyndallization, also known as fractional sterilization, is a sterilization method based on repeated cycles of heating at relatively low temperatures, separated by incubation periods. During each heating cycle, vegetative microorganisms are destroyed, while bacterial spores survive. During the subsequent incubation period, the spores germinate into vegetative cells, which are then eliminated during the next heating cycle. Repetition of this process over several days results in the destruction of both vegetative cells and spores (World Health Organization, 2024; International Organization for Standardization, 2018).

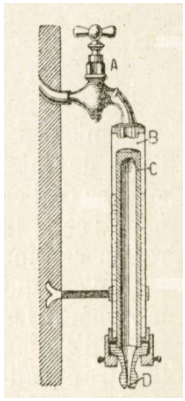
The classical tyndallization procedure consists of heating the material at 100°C for 20–30 minutes on three consecutive days, with 24-hour incubation periods at room temperature or at an appropriate incubation temperature between heating cycles to allow spore germination. Tyndallization is used only in exceptional circumstances for sterilizing heat-sensitive culture media or biological materials that cannot withstand the high temperatures of autoclaving and are unsuitable for membrane filtration. Examples include culture media containing serum, egg, gelatin, or certain sugars. Although effective when properly performed, tyndallization is time-consuming, less reliable than autoclaving, and has largely been replaced by modern sterilization techniques in routine microbiological practice (World Health Organization, 2024; International Organization for Standardization, 2018).

IX.5. Sterilization by filtration

Sterilization by filtration is a physical method used to remove microorganisms from heat-sensitive liquids that cannot be sterilized by heat. Unlike thermal sterilization methods, filtration does not destroy microorganisms but physically removes them by passing the liquid through a membrane with pores of a defined size (World Health Organization, 2024; International Organization for Standardization, 2018).

Sterile filtration is widely used for the preparation of heat-labile solutions such as vitamin solutions, antibiotics, sera, vaccines, enzymes, tissue culture media, and certain pharmaceutical products. It is suitable only for low-viscosity liquids that can readily pass through the filter membrane.

Several types of filters have been used in microbiology, including porcelain candle filters (Chamberland filters), sintered glass filters, and disposable membrane filters made of cellulose acetate, cellulose nitrate, polyethersulfone (PES), or polyvinylidene fluoride (PVDF). Today, disposable membrane filters are the most commonly used.



Chamberland filters



Porous glass filters



Filter membranes

Figure 10: Sterilization filters **(World Health Organization, 2024; International Organization for Standardization, 2018).**

For sterilizing aqueous solutions, membrane filters with a pore size of 0.22 µm are considered the standard because they effectively retain bacteria and fungi. Filters with a pore size of 0.10 µm may be used when the removal of very small bacteria (e.g., Mycoplasma spp.) is required.

Conventional membrane filtration does not reliably remove viruses, endotoxins, or soluble microbial toxins, as many viruses are smaller than the membrane pores and may pass through the filter (World Health Organization, 2024; International Organization for Standardization, 2018).

IX.6. Sterilization by radiation

Radiation sterilization is a physical method that uses electromagnetic or particulate radiation to eliminate or inactivate microorganisms. Depending on the energy of the radiation, sterilization methods are classified as ionizing or non-ionizing radiation (International Atomic Energy Agency, 2023).

IX.6.1. Ionizing radiation

Ionizing radiation includes gamma rays, X-rays, and electron beams, all of which possess sufficient energy to ionize atoms and molecules within microbial cells. These radiations destroy microorganisms by causing irreversible damage to DNA and other essential cellular components, thereby preventing microbial replication.

Ionizing radiation has excellent penetrating power and is widely used for the industrial sterilization of disposable medical devices, surgical instruments, syringes, catheters, Petri dishes, pharmaceutical products, and certain food products. Because of the specialized equipment required and the potential hazards associated with ionizing radiation, this sterilization method is restricted to industrial facilities operating under strict safety regulations.

IX.6.2. Non-ionizing radiation (Ultraviolet radiation)

Ultraviolet (UV) radiation is a non-ionizing form of electromagnetic radiation with wavelengths ranging from 100 to 400 nm. The UV-C region (200–280 nm), particularly at 254 nm, possesses the greatest germicidal activity because it induces DNA damage through the formation of pyrimidine dimers, thereby inhibiting microbial replication.

Ultraviolet radiation is commonly used for the disinfection of laboratory work surfaces, biological safety cabinets, laminar airflow cabinets, air, and water. However, UV radiation has poor penetrating ability and is effective only on surfaces or in transparent liquids that are directly exposed to the radiation. Consequently, UV treatment is considered a disinfection

method rather than a reliable sterilization method, as it cannot ensure complete destruction of all microorganisms, particularly bacterial spores located in shaded areas or beneath surfaces.

IX.7. Disinfection

Disinfection is the process of eliminating or inactivating most pathogenic microorganisms present on inanimate surfaces and objects. Unlike sterilization, disinfection does not necessarily destroy all microorganisms, particularly bacterial spores, although some high-level disinfectants may exhibit sporicidal activity under specific conditions (World Health Organization, 2024; Centers for Disease Control and Prevention, & National Institutes of Health, 2020; Centers for Disease Control and Prevention, 2019).

Disinfection is achieved through the application of chemical disinfectants to laboratory equipment, work surfaces, instruments, and environmental surfaces to reduce the microbial load and minimize the risk of infection and cross-contamination.

Chemical agents used to control microorganisms are classified according to their intended use (World Health Organization, 2024; Centers for Disease Control and Prevention, & National Institutes of Health, 2020; Centers for Disease Control and Prevention, 2019) :

- Antiseptics are antimicrobial agents with relatively low toxicity that are applied to living tissues, such as skin and mucous membranes, to reduce or inhibit the growth of microorganisms. Common examples include 70% ethanol or isopropyl alcohol, chlorhexidine, povidone-iodine, and hydrogen peroxide formulations intended for skin antisepsis.
- Disinfectants are chemical agents intended for use on inanimate objects and environmental surfaces. They generally possess greater antimicrobial activity and toxicity than antiseptics and are used to disinfect laboratory benches, instruments, floors, and equipment. Common disinfectants include sodium hypochlorite (bleach), phenolic compounds, quaternary ammonium compounds, glutaraldehyde, peracetic acid, and hydrogen peroxide.

The selection of an appropriate disinfectant depends on the type of microorganism present, the nature of the contaminated surface, the concentration of the disinfectant, the required contact time, and the presence of organic matter, which may reduce the effectiveness of some disinfectants.

IX.7.1. Liquid Disinfectants

Liquid disinfectants are widely used in microbiology laboratories to reduce microbial contamination on work surfaces, laboratory equipment, and reusable instruments. Their effectiveness depends on the disinfectant type, its concentration, the contact time, and the presence of organic matter (Centers for Disease Control and Prevention, 2019).

The most commonly used liquid disinfectants include:

- Alcohols (70% ethanol or 70% isopropyl alcohol): These are intermediate-level disinfectants used for the rapid disinfection of laboratory benches, small instruments, and other non-critical surfaces. Alcohols are effective against vegetative bacteria, fungi, and many enveloped viruses but are not sporicidal.
- Sodium hypochlorite (bleach): This chlorine-releasing disinfectant is commonly used for the decontamination of laboratory benches, floors, biological spills, reusable glassware, and contaminated materials. For routine laboratory disinfection, a freshly prepared solution containing 0.1% (1,000 ppm) available chlorine is generally recommended, whereas 0.5% (5,000 ppm) available chlorine is used for blood spills and heavily contaminated materials. Contaminated slides, Pasteur pipettes, and other reusable items may be immersed in an appropriate sodium hypochlorite solution before cleaning and sterilization.
- Soaps and detergents: Although soaps and detergents are not disinfectants, they are essential cleaning agents that remove dirt, organic matter, and a large proportion of microorganisms from surfaces. Cleaning should always precede disinfection because organic material can reduce the effectiveness of many disinfectants.

IX.7.2. Gaseous Disinfectants

Gaseous disinfectants are chemical agents used for the decontamination of enclosed spaces, laboratory equipment, and heat- or moisture-sensitive medical devices. Because many of these gases are toxic, irritant, or potentially carcinogenic, their use is restricted to controlled environments with appropriate ventilation and safety measures (World Health Organization, 2024; Centers for Disease Control and Prevention, 2019).

The principal gaseous disinfectants include:

- Formaldehyde (formalin): Formaldehyde gas, generated by heating formalin (an aqueous solution containing approximately 37–40% formaldehyde), has historically been used for the fumigation and decontamination of laboratories, biosafety cabinets, incubators, and enclosed rooms. Due to its toxicity and carcinogenicity, its use has largely been discontinued and replaced by safer alternatives such as vaporized hydrogen peroxide.

- Ammonia: Ammonia is not a disinfectant. Historically, it has been used after formaldehyde fumigation to neutralize residual formaldehyde vapors and reduce their irritant effects before personnel re-enter the treated area.

- Ethylene oxide (EtO): Ethylene oxide is a highly effective gaseous sterilant used primarily in industrial and hospital settings for sterilizing heat- and moisture-sensitive medical devices, including plastic equipment, catheters, syringes, electronic instruments, and packaged disposable materials. Ethylene oxide is capable of destroying bacteria, fungi, viruses, and bacterial spores. Because it is toxic, flammable, and classified as a human carcinogen, its use requires specialized equipment, strict process controls, and adequate aeration of sterilized materials before use.

PW 2 :

- ✓ ***Culture media***
- ✓ ***Inoculation techniques***
- ✓ ***Detection of laboratory microorganisms***







Part I: Culture medium

I.1. Introduction

Through this practical work, students become familiar with the different types of culture media, their composition, and the culture media types to choose for a specific purpose (isolation of bacteria from the total aerobic or anaerobic flora, pathogenic bacteria isolation, lactic acid bacteria isolation, yeasts and molds isolation, etc.). Thus, students will learn about the different inoculation types used in microbiology and when to use them according to the microbiological analysis to be performed.

I.2. Objectives

- To understand the different culture media used in microbiology.
- To understand the culture media composition and the role of each ingredient.
- To understand the culture media types used in microbiology.
- To be able to select and choose a culture medium according to the microbiological analysis to be performed.

I.3. Definition of culture media

A culture medium is a solid, semi-solid, or liquid nutrient preparation specifically formulated to support the growth, isolation, cultivation, identification, transport, or preservation of microorganisms under controlled laboratory conditions. Culture media provide the essential nutrients and environmental conditions required for microbial metabolism, growth, and reproduction (Manual of Clinical Microbiology, 2023).

I.4. Culture media main ingredients

The following table summarizes the different basic ingredients in a culture medium.

Table 1: Main ingredients of culture media.

Ingredients	Characteristics and functions
Meat extract	▪ Meat extract obtained through cold or hot maceration (infusion). ▪ Source of protein, carbohydrates, minerals, and water-soluble vitamins.
Yeast extract	▪ Baker's or brewer's yeast. ▪ Source of amino acids and water-soluble vitamins (vitamin B).
Peptone	▪ Action of proteolytic enzymes (pancreatin, pepsin, trypsin, etc.) on protein materials (meat, casein, soy, gelatin, etc.). ▪ Source of amino acids and oligopeptides.
Agar-agar or agar	▪ Red algae extract containing two polysaccharides' groups: agarose (70%) and agaropectin (30%). ▪ It dissolves in water at 90°C, remains supercooled at 45°C, and forms a gel at lower temperatures. ▪ Its function is to solidify culture media.
Biological products	Blood, serum, milk, gelatin, potato, etc.
Mineral base	▪ Ionic elements (Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, PO_4^-, SO_4^-, Zn, Cu, Fe). ▪ Maintain electrical and osmotic balance.
Distilled water	

To support optimal microbial growth, a culture medium should provide (Manual of Clinical Microbiology, 2023; American Society for Microbiology, 2023) :

- Water, which serves as the solvent for biochemical reactions.
- Carbon and energy sources, such as carbohydrates or organic compounds.
- A nitrogen source, required for the synthesis of proteins, nucleic acids, and other cellular constituents.
- Mineral salts and trace elements, including phosphorus, sulfur, magnesium, potassium, calcium, and iron, which are essential for cellular metabolism.

- Growth factors, such as vitamins, amino acids, purines, or pyrimidines, required by fastidious microorganisms.
- An appropriate pH, generally close to the optimum growth pH of the microorganism being cultivated (typically around pH 7.0 for most bacteria).
- Suitable atmospheric conditions, including oxygen, carbon dioxide, or anaerobic conditions, depending on the physiological requirements of the microorganism.

The composition of a culture medium varies according to its intended purpose, such as general cultivation, selective isolation, differential identification, enrichment, transport, or preservation of microorganisms.

Table 2: Characteristics of Common Selective and Differential Culture Media.

Culture Medium	Purpose	Typical Colony Appearance
Chapman Agar (Mannitol Salt Agar, MSA)	Selective and differential medium for the isolation of *Staphylococcus* spp.	*Staphylococcus aureus* ferments mannitol, producing yellow colonies surrounded by a yellow zone. Non-mannitol-fermenting staphylococci (e.g., *S. epidermidis*) produce pink to red colonies, with no color change in the medium.
Hektoen Enteric (HE) Agar	Selective and differential medium for the isolation of *Salmonella* and *Shigella* species from clinical and food samples.	*Salmonella* spp. produce blue-green colonies with black centers (H_2S production). *Shigella* spp. produce green colonies without black centers. Lactose-fermenting bacteria produce yellow to orange colonies.
Salmonella–Shigella (SS) Agar	Selective and differential medium for the isolation of *Salmonella* and *Shigella* species.	*Salmonella* spp. produce colorless colonies with black centers due to H_2S production. *Shigella* spp. produce colorless colonies without black centers. Lactose-fermenting bacteria produce pink to red colonies.
Eosin Methylene Blue (EMB) Agar	Selective and differential medium for Gram-negative enteric bacteria based on lactose fermentation.	*Escherichia coli* produces dark blue-black colonies with a characteristic metallic green sheen. Other lactose fermenters produce pink to purple colonies, whereas non-lactose fermenters produce colorless or translucent colonies.

I.5. Culture media classification

A. According to the composition

Synthetic media	Semi-synthetic media	Natural or empirical environments
The composition is known both qualitatively and quantitatively. Used to study the nutritional requirements of a germ (e.g., urea-tryptophan, Simmons citrate).	Synthetic media supplemented with an extract of organism such as yeast extract containing growth factors for bacteria (amino acids + vitamins) or a peptone (e.g., glycerol nitrate agar used for the determination of *Penicillium* species).	The composition is known only approximately because it depends on the raw materials used: meat or yeast extracts, peptones, sugars and possibly biological liquids (serum, blood...).

- **Determination of coagulase in Staphylococcus aureus:**

  Bacterial suspension + oxalate serum ===> Incubation 37°C/4h ==> Observation of the coagulation reaction until the 4th hour.

- **Determination or investigation of blood hemolysis:**

Inoculation of the bacterial suspension onto 5% human or horse blood agar and observation of the blood hemolysis areas: clear area around the colonies==>**β-hemolysis** or **complete hemolysis**, greenish area (green color due to the presence of biliverdin which is an intermediate of heme degradation)==>**α-hemolysis** or **partial hemolysis**, no area==>**γ-hemolysis** or **absence of hemolysis**.

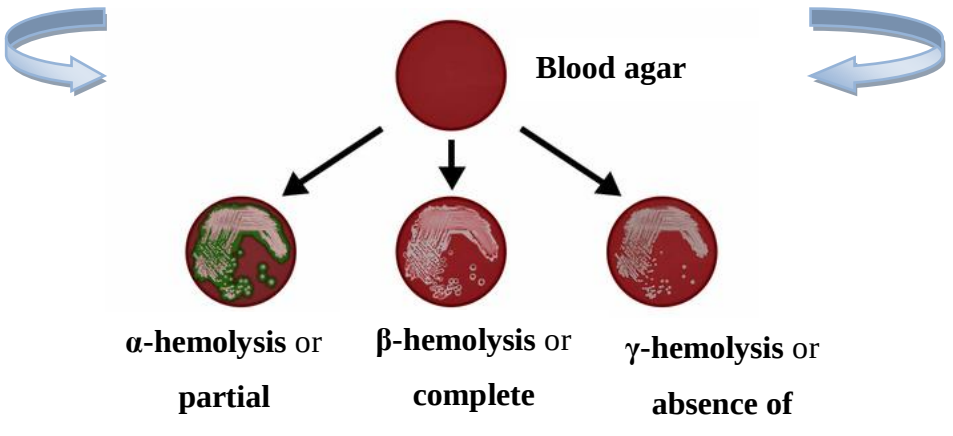


Figure 11: Classification of culture media according to composition.

B. According to use

General purpose culture media

- Supports the growth of various microorganisms:
- ✓ **Tryptic Soy Agar**.
- ✓ **Nutrient broth** that allows for the microorganisms revitalization.
- ✓ **Nutrient agar** for the isolation of total aerobic flora.
- ✓ **Colombia Agar** for the isolation of total anaerobic flora.

Enriched culture media

Added to compounds of higher nutritional value (blood, serum, multivitamin supplements) (E.g. **blood agar**, **milk agar**).

Differential culture media

They promote the growth of certain specific microorganisms while inhibiting the growth of other species or isolates.

MacConkey agar: ***Enterobacteriaceae*** isolation (Gram-negative bacilli) ==> this medium contains lactose as a carbon source. Therefore, *Enterobacteriaceae*, coliforms from water and food products, break down lactose, resulting in pink colonies.

Hecktoen agar: Selective and differential medium for the Gram-negative bacilli of the ***Enterobacteriaceae*** family isolation, particularly the isolation of ***Salmonella*** and ***Shigella*** species from fecal samples (mixed flora).

The **selectivity** is due to the presence of **bile salts** and **dyes** that inhibit most Gram-positive bacteria.

The **differentiation**: carbohydrates fermentation (lactose, sucrose, and salicin) ==> orange-yellow colonies.

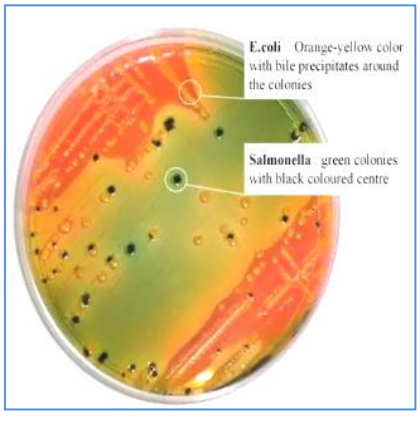
Bromothymol blue, after culture medium acidification, turns yellow. In the presence of acids, it reacts with fuchsin, resulting in a red coloration.

Escherichia coli: orange-yellow colonies with a bile precipitate surrounding the colony.

Salmon-colored colonies: ***E. coli***, ***Enterobacter***, ***Klebsiella***, ***Proteus***.

Green/blue-green colonies: ***Proteus*** with or without a black center, ***Salmonella***, ***Shigella***, ***Pseudomonas*** (green to brown).

Gram-positive bacilli: no growth.



Salmonella and ***Shigella*** are unable to use the three carbohydrates present in Hecktoen agar, resulting in translucent, light green, or bluish-green colonies. While most non-pathogenic coliforms can use at least one sugar.

Differentiation relies on the **hydrogen sulfide production**, made possible by the presence of sodium thiosulfate and iron citrate, leading to the iron sulfide formation.

This results in black colonies or colonies with black centers, indicating ***Salmonella***.

Green colonies without a black center indicate ***Shigella***.

B. According to use

- **General purpose culture media**
- **Enriched culture media**
- **Differential culture media**
- **Conservation culture media**

***Salmonella-Shigella* agar**: The presence of lactose and a neutral red color allows to know the lactose character of bacteria.

Pink/red colonies indicate a medium acidification (the bacteria are therefore using lactose present in the SS-agar as carbon and energy source: lactose positive).

Colorless or yellow colonies indicate an acidification absence (the bacteria are therefore no longer using lactose: lactose negative).

Sodium thiosulfate is a source of sulfur from which certain bacteria form **hydrogen sulfide (H_2S)**, which reacts with ferric ions to form an iron sulfide black precipitate.

Salmonella: colorless colonies with or without a black center (lactose-, H_2S-positive or H_2S-negative).

Shigella: colorless colonies without a black center (lactose-, H_2S-).

Coliforms (***E. coli***): pink/red colonies (lactose-, H_2S-).

Low nutrient agar or minimal media (nutrient-poor culture media) that maintain bacteria in slowed life state (e.g., for fastidious bacteria, a liquid medium of BGT (Buffered Glucose Broth) + Glycerol is used, which is frozen at -80°C).

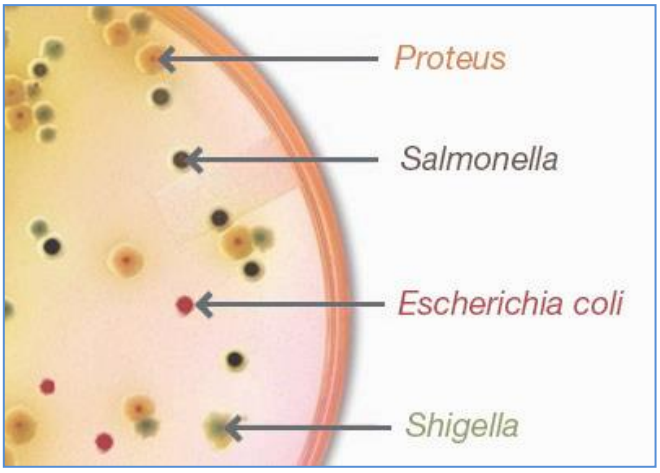


Figure 12: Culture media classification according to use (A-B).

Table 3: Mac Conkey agar composition.

Mac Conkey agar composition	
Carbon and energy source	Lactose
Amino acids, sulfur and phosphorus source	Gelatin pancreatic hydrolysate, casein or pepsin hydrolysates of animal tissues.
Inhibitors	Bile salts, violet crystal.
Na^+ source	NaCl (to maintain balanced osmotic pressure).
pH indicator	Neutral red.
Solidifying agent	Agar-agar.
Distilled water	
Final pH at 25°C, pH = 7.1 ±0.2.	

Table 4: Hecktoen agar composition.

Hecktoen agar composition	
Source de carbone et d'énergie	Proteose-peptone
Growth factor	Yeast extract
Differentiation criterion	Lactose, sucrose, salicin
Revealing H_2S	Iron III ammonium citrate.
Inhibitor	Bile salts, acid fuchsine
pH indicator	Bromothymol blue.
Maintains osmotic pressure	Sodium chloride.
Precursor of H_2S	Sodium thiosulfate.
Solidifying agent	Agar-agar.
Eau distillée	
pH final à 25°C, pH = 7.5 ±0.2.	

Tableau 5: *Salmonella-Shigella* agar composition.

Salmonella-Shigella agar composition	
Source de carbone et d'énergie	Proteose-peptone
Differentiation criterion	Lactose
Growth factor	Beef extract
Inhibitor	Bile salt,
Inhibitor	Sodium citrate
Inhibitor	Brilliant green
Precursor of H_2S	Sodium thiosulfate
Revealing H_2S	Ferric citrate
pH indicator	Neutral red
Solidifying agent	Agar-agar.
Eau distillée	
pH final à 25°C, pH = 7 ±0.2.	

Part II: Culture media preparation

II. Culture media preparation steps

The main steps in preparing a culture medium are (Manual of Clinical Microbiology, 2023):

1. Weighing the different components.
2. Dissolving the medium: dissolving the medium ingredients in beakers with twice the volume of the prepared solution. Stir until the components are completely dissolved.
3. Adjusting the pH by adding sterile sodium hydroxide (NaOH) or hydrochloric acid (HCl).
4. Adding the solidifying agent (if required): Agar-agar.
5. Dispensing the medium: Distribute the prepared medium into clean culture tubes, flasks, or bottles, leaving sufficient headspace to allow expansion during sterilization.6. Sterilizing the culture medium (autoclaving at 121°C/20 min).
7. Sterilization: Sterilize the prepared medium by autoclaving at 121°C under 15 psi (103 kPa) for 15–20 minutes, unless the medium contains heat-sensitive components that require filtration or aseptic addition after sterilization.
8. Cooling and pouring (for agar media): After autoclaving, allow the medium to cool to approximately 45–50°C before pouring it into sterile Petri dishes or adding heat-sensitive supplements such as antibiotics, blood, or vitamins.

9. Storage: Store prepared culture media under the recommended conditions, usually at 2–8°C, protected from light and dehydration. Before use, inspect the media for contamination, dehydration, color changes, or other signs of deterioration.

Part III: Techniques for inoculating different culture media

III. Inoculation Techniques according to culture media physical state

III.1. Liquid Media

Bacterial colony Inoculation: **Dissociate** in a liquid medium a bacterial colony taken with the inoculation loop platinum or the Pasteur pipette.

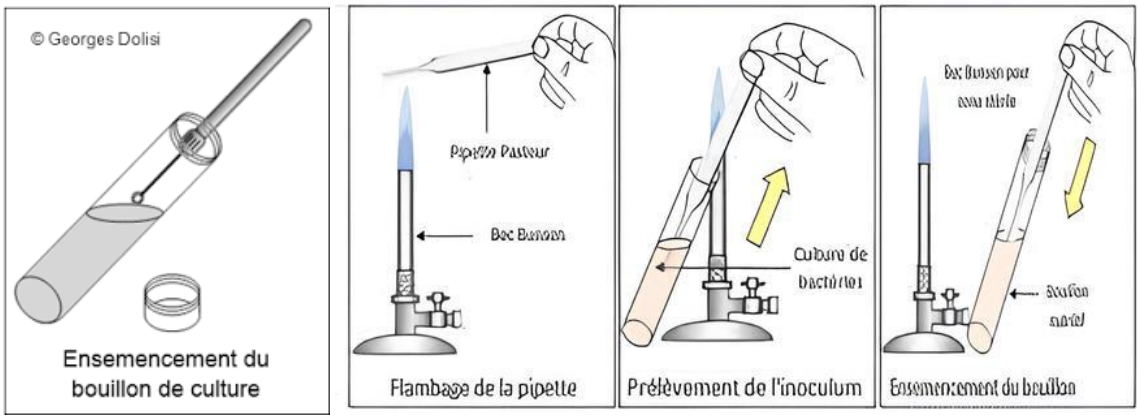


Figure 13: Inoculation technique in liquid medium.

III.2. Solid media in tubes

Method 1

- Inoculation of an agar in a tube by **central puncture** with the Pasteur pipette (E.g. **mannitol-mobility medium**):
- Ensure the semisolid agar medium is in a test tube, and you have a sterile Pasteur pipette ready for use.
- Carefully insert the sterile pipette, with the bacterial culture, straight down the center of the tube. Go about halfway to two-thirds of the way down, but avoid puncturing the bottom of the tube.

- Slowly and carefully pull the pipette out, following the exact same line it was inserted on. Avoid any fanning or swiping motions.
- Incubate the tube under the specified conditions (37°C for 18 to 24h).
- After incubation, hold the tube up to the light. A positive motility test will show diffuse, hazy growth that has spread away from the initial inoculation line. A negative result will have growth only along the sharp stab line.

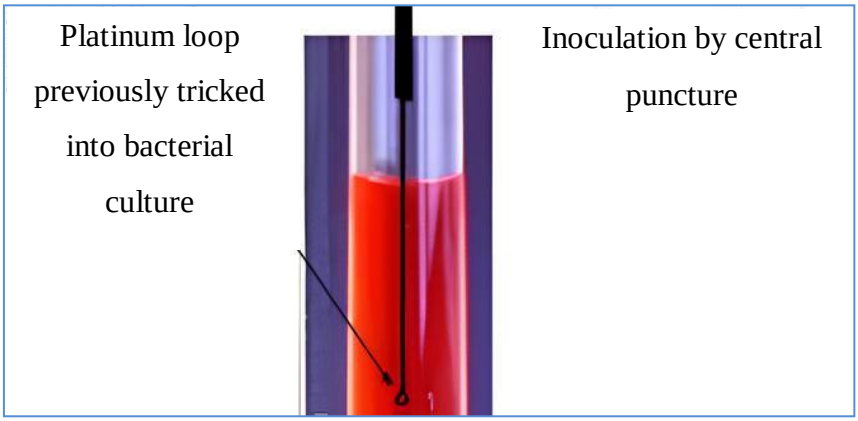


Figure 14: Inoculation of mannitol-mobility medium by central puncture (Deep inoculation).

Method 2

- Transverse streaking on a tube-shaped inclined agar or slant agar (E.g.. TSI medium: Triple Sugar Iron or Simmons citrate agar).

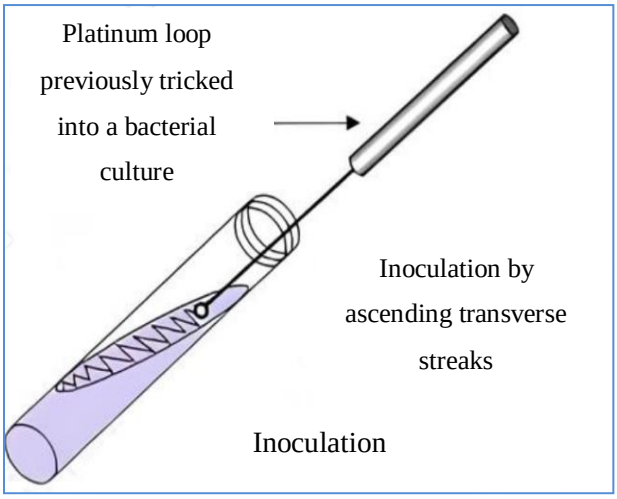


Figure 15: Inoculation of TSI medium (slant agar) by transverse streaks.

III.3. Solid media in Petri dishes

- **Streak inoculation**: The principle consists of exhausting an initial sample or pure bacteria deposit by applying the streak-plate method (through successive streaks) (Clinical and Laboratory Standards Institute, 2024; Manual of Clinical Microbiology, 2023).

Streak inoculation of a bacterial colony: The bacterial colony is spread over the entire surface of a solid culture medium by making close streaks at the beginning and farther apart towards the end using an inoculation loop platinum, a Pasteur pipette or a swab.

Quadrant-streak inoculation (exhaustion): Isolation may be considered to separate different microorganisms within a mixture, purify a contaminated strain, or control its purity.

- Draw two perpendicular diameters on the outer edge of the agar Petri dish, dividing the dish into four sections.
- Place the inoculum near one edge of the half of the dish corresponding to quadrants 1 and 2.
- Spread this deposit, creating closely spaced streaks (quadrants 1 and 2).
- Rotate the dish approximately 90° and spread a portion (quadrant 3) by streaking the corresponding half (quadrants 2 and 3).
- Rotate the dish approximately 90° again and repeat the same operation, applying streaks to the remaining half (quadrants 3 and 4).

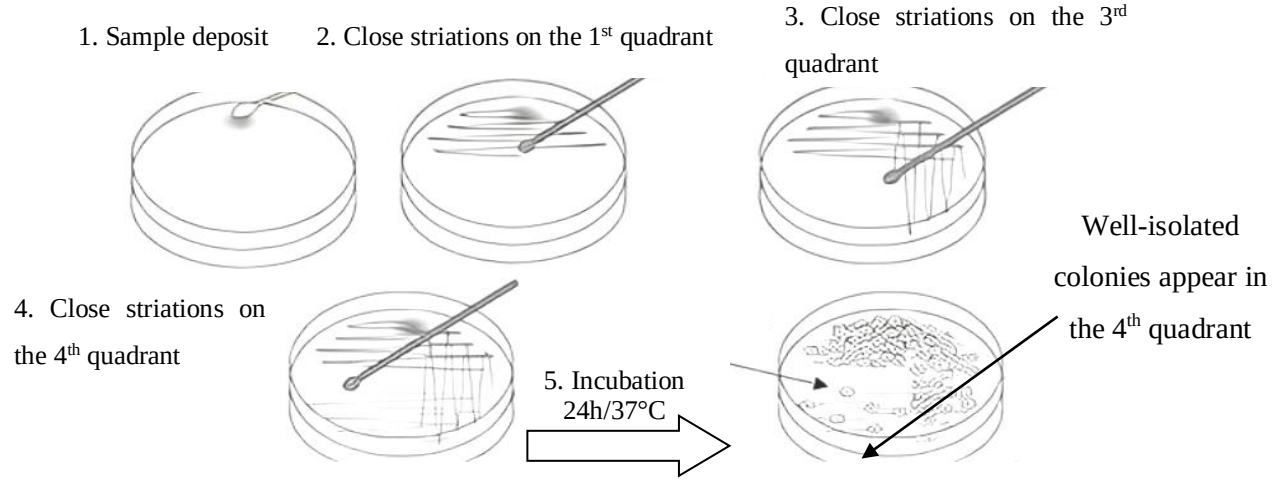


Figure 16: Inoculation of solid culture medium by streaking (quadrant-streak inoculation technique).

- ***Flood Inoculation (Flooding Method)***

Pour a sufficient volume of the microbial suspension onto the surface of the agar to completely cover it.

Gently tilt the plate to ensure uniform distribution of the inoculum over the entire surface.

Remove the excess suspension using a sterile pipette or by carefully decanting.

Allow the agar surface to dry under aseptic conditions (or according to the protocol) before incubating under appropriate conditions.

Application: This method is commonly used for bacteriophage assays, surface contamination studies, and some antimicrobial susceptibility testing protocols (Clinical and Laboratory Standards Institute, 2024; Manual of Clinical Microbiology, 2023).

- **Spreading Inoculation (Spread Plate Method)**

Pipette 0.1 mL of the microbial suspension or appropriately diluted sample onto the surface of a pre-solidified agar plate.

Using a sterile L-shaped spreader (hockey-stick spreader), evenly distribute the inoculum over the entire agar surface.

Allow the inoculum to be absorbed into the agar before inverting and incubating the plate under appropriate conditions.

Application: This method is commonly used for the enumeration of viable microorganisms and the isolation of surface colonies (Manual of Clinical Microbiology, 2023).

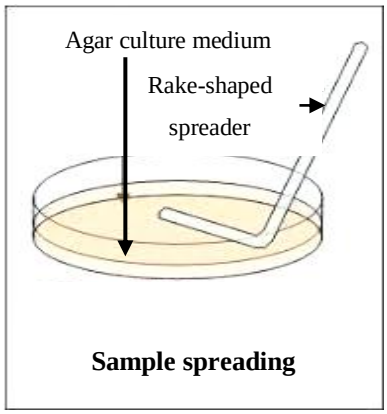


Figure 17: Inoculation by spreading on the solid culture medium surface.

- **Swab Inoculation (Lawn Culture Method)**

Dip a sterile cotton swab into the microbial suspension and remove excess liquid by pressing it against the inside wall of the tube.

Evenly spread the inoculum over the entire surface of the agar by swabbing in three directions, rotating the plate approximately 60° between streaks to obtain a uniform bacterial lawn.

Allow the surface to dry for 3–5 minutes before performing subsequent procedures (e.g., antibiotic disc placement for the Kirby–Bauer antimicrobial susceptibility test) (Clinical and Laboratory Standards Institute, 2024; Manual of Clinical Microbiology, 2023).

Spreading the bacterial suspension over the entire agar surface by swabbing.

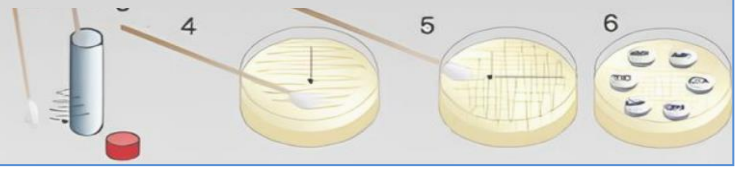


Figure 18: Inoculation by swabbing the solid culture medium surface.

- **Deep Inoculation (Pour Plate Method)**

The pour plate method (deep inoculation) is a microbiological technique used to enumerate viable microorganisms or to isolate colonies distributed throughout a solid culture medium (International Organization for Standardization, 2013).

The procedure is performed as follows:

⇨ Transfer 1.0 mL of the microbial suspension or appropriately diluted sample into a sterile Petri dish.
⇨ Pour 15–20 mL of molten agar medium, previously cooled to 45–50°C, into the Petri dish. Gently mix the inoculum with the medium by carefully rotating the plate in a figure-eight (∞) or circular motion to ensure uniform distribution of the microorganisms throughout the agar.
⇨ Allow the agar to solidify completely on a level surface at room temperature.

⇨ Once solidified, invert the Petri dishes and incubate them under appropriate conditions (temperature, atmosphere, and incubation time) according to the growth requirements of the target microorganisms.

This technique allows microorganisms to develop both within the agar and on its surface, making it particularly useful for the enumeration of viable bacteria in food, water, environmental, and clinical samples.

Partie IV: Bacteria isolation

IV.1. Objectives

- Apply the different inoculation techniques studied previously.
- Isolate bacteria from airborne flora, skin flora, and flora on undisinfected surfaces by applying the different inoculation techniques (flooding, spreading, swabbing, quadrant-streak inoculation, streak inoculation).
- Be able to describe the bacterial colonies macroscopic appearance that appear on inoculated culture media (Part V).

IV.2. Materials

A. Culture Media

- Nutrient Agar (NA): total aerobic flora isolation (mixed flora ====> Gram-positive and Gram-negative bacteria).
- Chapman Agar: *Staphylococcus* isolation (e.g., *Staphylococcus aureus*, *S. epidermidis*) =======> Gram-positive cocci.
- Eosin Methylene Blue Agar (EMB): *Enterobacteriaceae* isolation (e.g., *Escherichia coli, Enterobacter sp., Proteus sp., Klebsiella sp*) =====> Gram-negative bacilli.

B. Consumable materials: Petri dish, Pasteur pipette, Swab, Sterile tip, Syringes.

C. Glassware: Sterile test tubes.

D. Lab Equipment: Vortex mixer, Electronic balance, Stirrer, Water bath, Incubator, Refrigerator.

F. Other: Inoculation loop platinum, Micropipette, Test tube rack.

IV.3. Protocol

To isolate bacteria from different samples, the following steps must be followed:

1. Poor the culture media onto Petri dishes.

2. Inoculate the culture media using the various techniques described in Table 5.

3. Incubate the bacterial cultures at 37°C for 18–24 hours.

4. Perform a macroscopic examination of the bacterial colonies appeared on the different culture media used (macroscopic characterization): Observe and describe their visual appearance (size, shape, surface area, opacity, consistency, and color (pigmentation)) (Table 6–Figure 9).



Figure 19: Typical Colony Morphology on Eosin Methylene Blue (EMB) Agar and Chapman Agar.

Table 6: Inoculation techniques applied for the bacteria isolation.

Samples	**Inoculation methods**	**Culture media**	**Temperature and incubation time**
Tap water	Flood inoculation	Nutrient agar	Incubation at 37°C/18-24h
	Surface spreading inoculation		
	Deep inoculation		
Air	Agar direct contact with the air circulating in the laboratory		
Hands	Swabbing	Chapman agar	
Laboratory door handle	Swabbing	Chapman agar	
Soil sample	Quadrant-streak inoculation	EMB agar	
	Streak-plate inoculation		

Table 6: Characteristics to be evaluated after bacterial colonies appearance on the different culture media.

Criteria	**Description**
Size	Small, medium or large.
Shape	Round, irregular, star-shaped, etc. The outline can be smooth or jagged.
Surface	Smooth, rough, or displaying patterns like ripples.
Opacity	Opaque, translucent or transparent.
Consistency	Oily, creamy, dry or mucous.
Pigmentation or color	The color can vary considerably (whitish, orange, golden yellow, etc.).

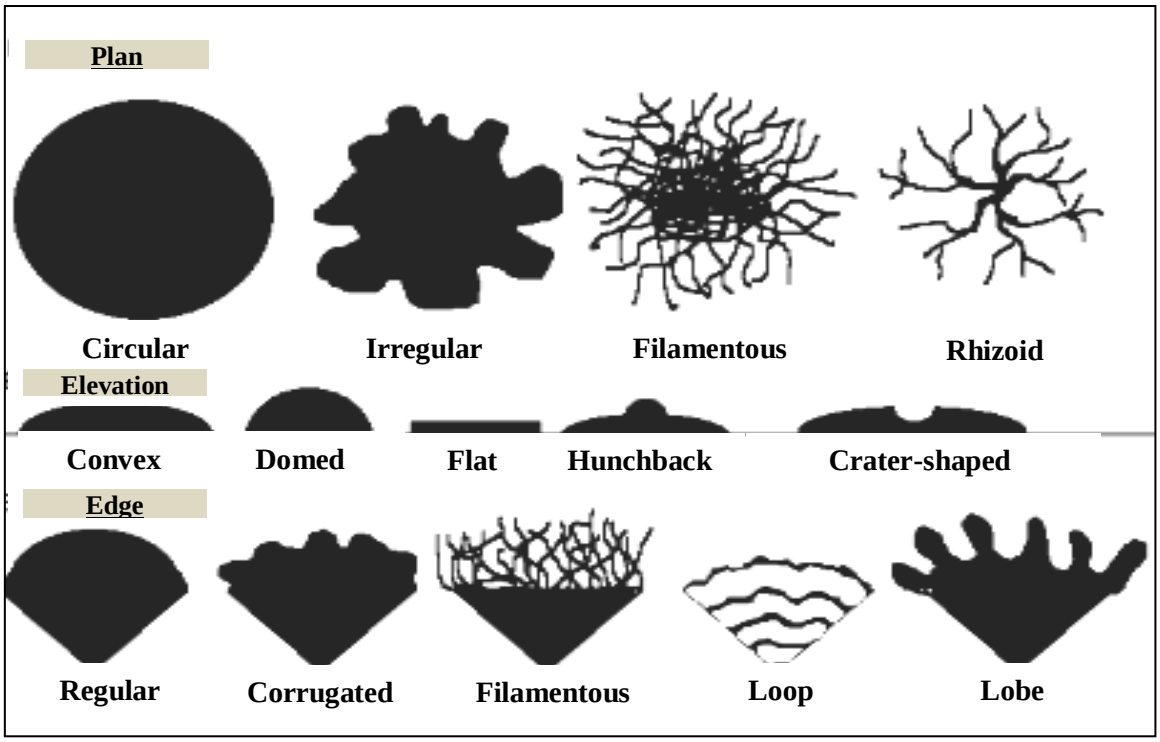
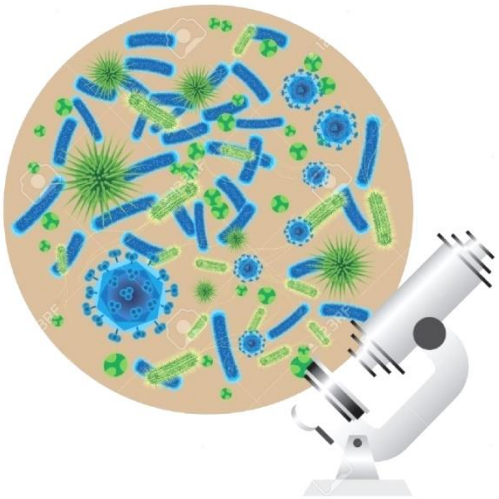


Figure 20: Colony morphologies.

PW 3 : Microscopic examination of bacteria: Wet mount and Gram differential staining



I. Introduction

Through this practical work, students will perform a microscopic examination of bacteria previously isolated on different culture media as a follow-up to bacterial identification. Initially, they determine the appearance of bacterial colonies that have appeared on culture media as a first step in bacterial identification through a macroscopic bacteria examination, subsequently, the students will perform a microscopic examination of the fresh specimen to observe the bacterial cells shape, their association mode, and bacterial motility.

In some microbiological analysis, the fresh state is crucial and serves as a key test in bacterial identification, taking as an example the identification and differentiation between *Salmonella* and *Shigella* colonies previously isolated on SS agar, *Salmonella* will give colorless colonies with or without a black center, while *Shigella* will give colorless colonies without a black center.

In the case of having the same microscopic appearance on SS agar (colorless colonies without a black center), the next step is microscopic examination by testing the bacterial cells fresh state, and as a first guideline, if motile bacteria are observed, it can be deduced that the colonies that appeared on the SS agar are *Salmonella*, and if non-motile bacteria are observed, it is therefore *Shigella*, and in the case of observation of a mixed culture of motile and non-motile bacteria, a bacterial colonies purification is so very important.

Thus, through this PW, students will learn how to perform a differential Gram stain in order to determine the bacteria Gram staining previously isolated on culture media.

II. Microscopic Examination Techniques

II.1. Fresh specimen examination

It allows the observation of living bacteria and the determination of their morphology, their grouping mode, their mobility and the approximate quantity of bacteria cells.

For the mobility assessment, it's sometimes necessary to use the bacterial mobility test, which can be carried out in mannitol-mobility-nitrates medium (Delarras, 2007).

II.1.1. Technique

Take a clean microscope slide, cleaned with a cotton ball soaked in 95% alcohol:

- For a culture in liquid medium (broth), place a drop of this culture onto the microscope slide using a sterile Pasteur pipette, or place an inoculum onto the slide using a platinum loop.
- For a culture on solid medium (agar) in a tube or Petri dish, first place a drop of sterile distilled water or sterile physiological saline onto the microscope slide, then add and dissolve a bacterial inoculum in the water.
- Cover with a coverslip, then seal the preparation with paraffin or petroleum jelly.
- Observe under a light microscope with a 40x or 60x objective. To highlight certain structural details, a 100x oil immersion objective should be used.

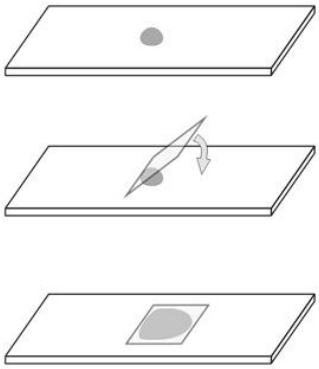


Figure 21: Bacterial fresh state examination procedure.

II.2. Gram stain

II.2.1. Discovery

Gram, Hans Christian Joachim (1853-1938), a Danish physician and bacteriologist, discovered in Berlin in 1884 a staining method that would bear his name, while he was working on pneumococci.

II.2.2. General principle

On the prepared bacterial smear, the first stain, oxalate crystal violet, will stain the bacteria violet, then Lugol's iodine (iodine-iodide solution) releases iodine which will fix the previous stain.

An iodine-violet crystal complex forms, which will be solubilized by 95°C alcohol during the decolorization phase, only for Gram-negative bacteria.

The second stain, called a contrast agent, safranin, will stain Gram-negative bacteria pink; Gram-positive bacteria, which are not decolorized by alcohol, retain their purple color.

The division of the bacterial world into two groups (Gram-positive and Gram-negative) is primarily due to a difference in the chemical structure of bacterial cell walls (see Chapter II - General Microbiology).

II.2.3. "Gram-Hucker" technique

II.2.3.1. Procedure

"Gram-Hucker" technique is an American technique. The Gram staining technique, modified by Hucker in 1902 and known as Gram-Hucker staining, is the subject of a standardized presentation in food microbiology (NF EN ISO 7218 standard, October 2007). The protocol is as follows:

- Prepare a smear of a pathological specimen or a pure bacterial culture.
- Cover the smear with oxalate crystal violet, leave for 1 minute, then rinse with distilled water.
- Add Lugol's iodine and leave for 1 minute, then rinse with distilled water.
- Decolorize with 95% alcohol for 15 to 30 seconds, then rinse with distilled water.
- Recolor with safranin for 10 to 30 seconds, then rinse with distilled water.
- Dry over the Bunsen burner flame.

Safranin can be replaced by Ziehl's fuchsin diluted 1/10 for 1 minute.

- Observe under a 100x immersion optical microscope.

With this dual staining, Gram-positive bacteria appear dark purple, while Gram-negative bacteria are stained pink or red.

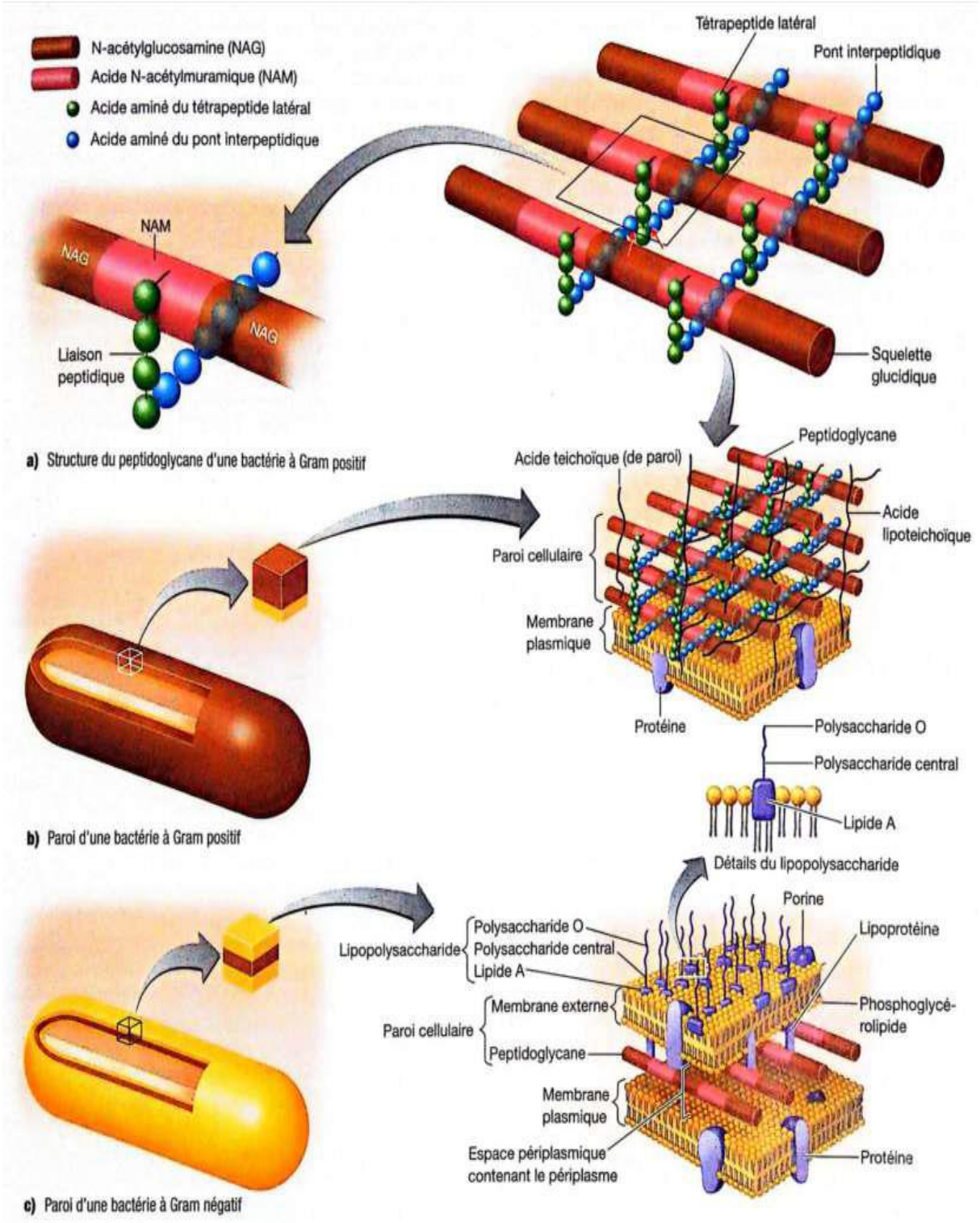


Figure 22: Gram-positive and Gram-negative bacterial wall structure (Meyer, 1994).

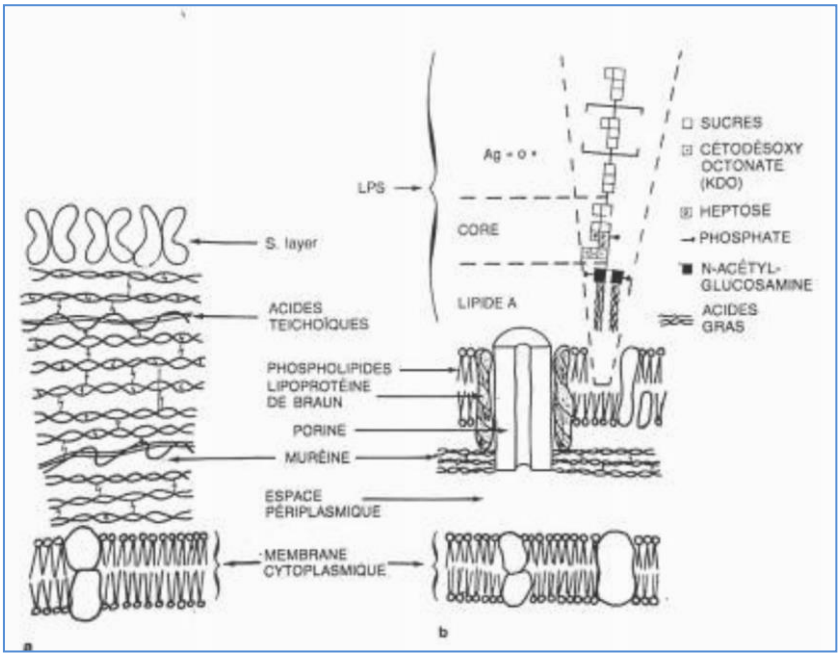


Figure 23: Diagram of cell envelopes. a: Gram-positive bacteria, b: Gram-negative bacteria (Meyer, 1994).

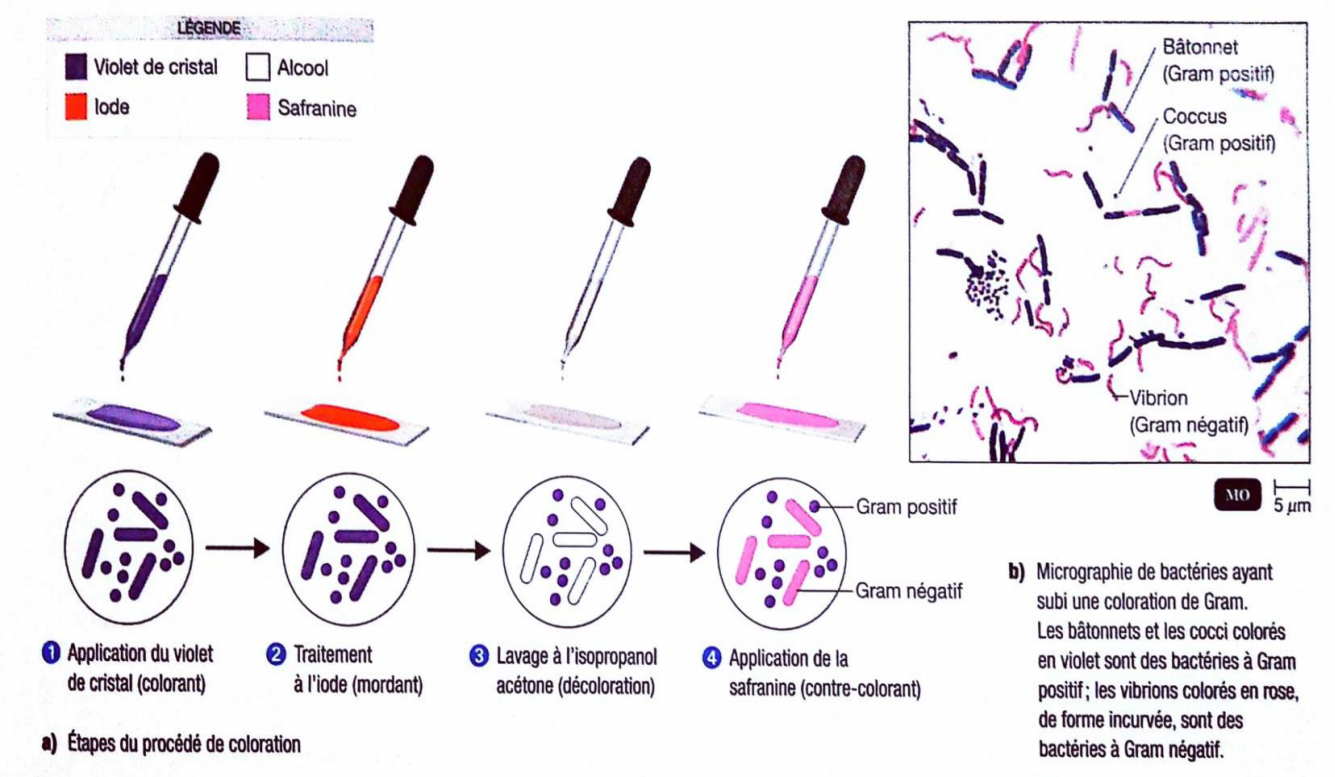


Figure 24: Gram staining (Martin, 2012).

II.2.3.2. Gram Hucker stains

The chemical composition of the stains used in Gram Hucker staining is shown in the tables below.

Table 8: Chemical composition of crystal violet.

Crystal violet solution	
Crystal violet	2 g
Ethanol	20 mL
Ammonium oxalate	0.8 g
Distilled water	80 mL

Table 9: Chemical composition of Lugol's iodine.

Lugol's iodine solution	
Iodine	1 g
Potassium iodide	2 g
Distilled water	80 mL

Table 10: Chemical composition of the safranine solution.

Safranine solution	
Safranine O	0.25 g
Ethanol 95%	10 mL
Distilled water	100 mL

PW 4: Bacterial identification and antibiogram





Bacterial identification and antibiogram

I. Introduction

Bacterial identification is used to give an identity to an unknown bacterium. Through this practical work, students will learn about the different biochemical and enzymatic identification tests that follow the identification steps previously carried out. A series of enzymatic and biochemical identification tests will be carried out using macro-methods and micro-methods to establish an identity for a bacterial strain that was initially unknown. Therefore, students will understand the interest and importance of these identification tests in microbiology because they serve as a crucial step in clinical orientation.

Students will therefore be trained on the different microbiological analyses carried out, whether in the medical field (medical analysis laboratories) or the agri-food field (microbiological quality control laboratories for food).

In the medical field, biochemical identification must be coupled with an antibiogram to determine the antibiotic resistance profile of the identified strain, which subsequently helps the doctor to prescribe antibiotics effective in killing the pathogenic bacterium causing illness or food poisoning.

II.1. Identification by macro-method

- ✓ Identification media are liquid and/or solid culture media that allow for biochemical tests relating to the respiratory, carbohydrate, protein and lipid metabolism of bacteria.
- ✓ These culture media in classic tubes and their biochemical tests form a biochemical gallery for the specific bacteria identification (*Enterobacteriaceae*, *Staphylococcus* species, *Micrococcus*...etc).
- ✓ These galleries, consisting of media in culture tubes and hemolysis tubes (macro-method), are of educational interest and useful for some microbiological analysis or control laboratories, but they are generally replaced by micro-galleries.

II.2. Identification by micro-method

The identification media are grouped in a micro-gallery, allowing several biochemical tests to be performed simultaneously. This is a micro-identification method using API systems (bioMérieux API gallery) (Delarras, 1981).

II.3. Biochemical tests

- ✓ Conventional biochemical tests of respiratory, carbohydrate or protein metabolism, for the bacteria differentiation or identification, are used in routine laboratory practice (Table 11).
- ✓ In microbiology, the reagents commonly used when reading biochemical tests involved in the bacterial metabolism study and in bacterial identification are mentioned below.

Table 11: API BioMerieux galleries and identifiable bacteria.

Galeries API	Nombre de tests biochimiques	Temps d'incubation en heures	Bactéries identifiées
API 20 A	20	24	Anaerobic bacteria: *Clostridium*
Fast ID 32 A2	29	4	
API 20 NE	20	24	Gram - bacilli non-enterobacteria
API 50 CH	50 cups ; 49 sugars	24 to 48	*Bacillus*
ID 32 GN	32	24	*Enterobacteriaceae* and other Gram - bacilli (*Pseudomonas*)
API Campy	20	24	*Campylobacter*
API 10 S	11	18 to 24	*Enterobacteriaceae* and other Gram - bacilli
API 20 E	20	18 to 24	*Enterobacteriaceae*
ID 32 E	32	24	*Enterobacteriaceae* and other Gram - bacilli
Rapid 32 E	32	4	*Enterobacteriaceae*
API 20 C AUX	20 cups ; 19 tests	48 to 72	Yeasts
ID 32 C	32	24	

Table 11 (continued)			
API Candida	12	18 to 24	*Candida* (yeast)
API Listeria	10	18 to 24	*Listeria*
API Staph	20	18 to 24	Staphylococci, micrococci and related germs
ID 32 Staph	32 cups; 26 tests	24	Staphylococci, micrococci and related germs
API Strep	20	4 then 24	*Streptococcaceae* and related germs
Fast ID 32 Strep	32	4	

Table 12: Biochemical tests.

Metabolism	Biochemical tests
Respiratory	Catalase test
	Oxidase test
	Nitrate reductase test
	ML test
	SMCMP test
	Clark et Lubs test
Carbohydrate	ONPG-hydrolase test
	TSI/KIA test
	Simmons citrate test
	Esculina test
	SMCMP test
	Mannitol-mobility test
Protein	Indole test, LDC test, ODC test, ADH test, H_2S test, free coagulase test

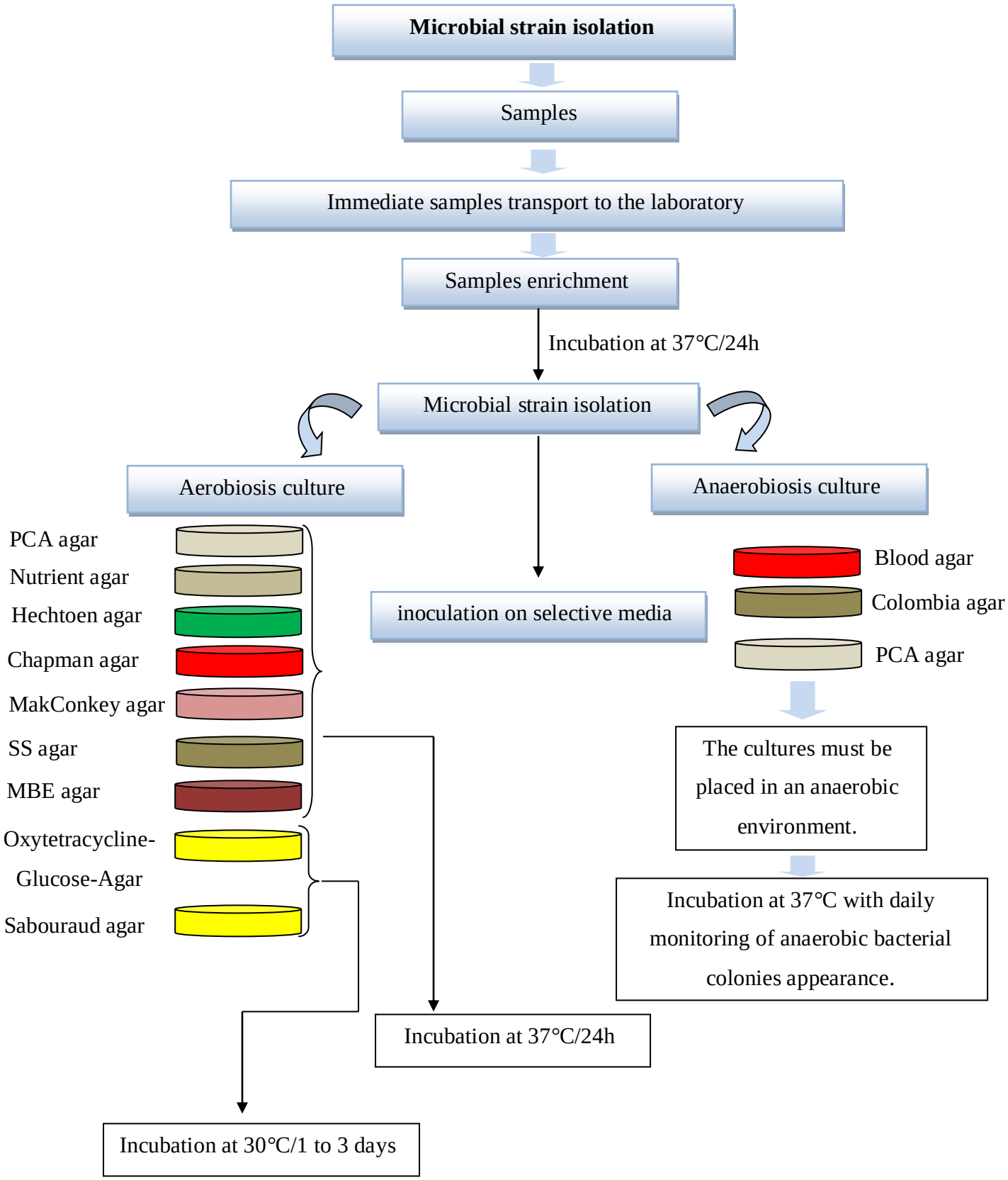


Figure 25: Summary diagram of the main steps to follow during microbiological analyses.

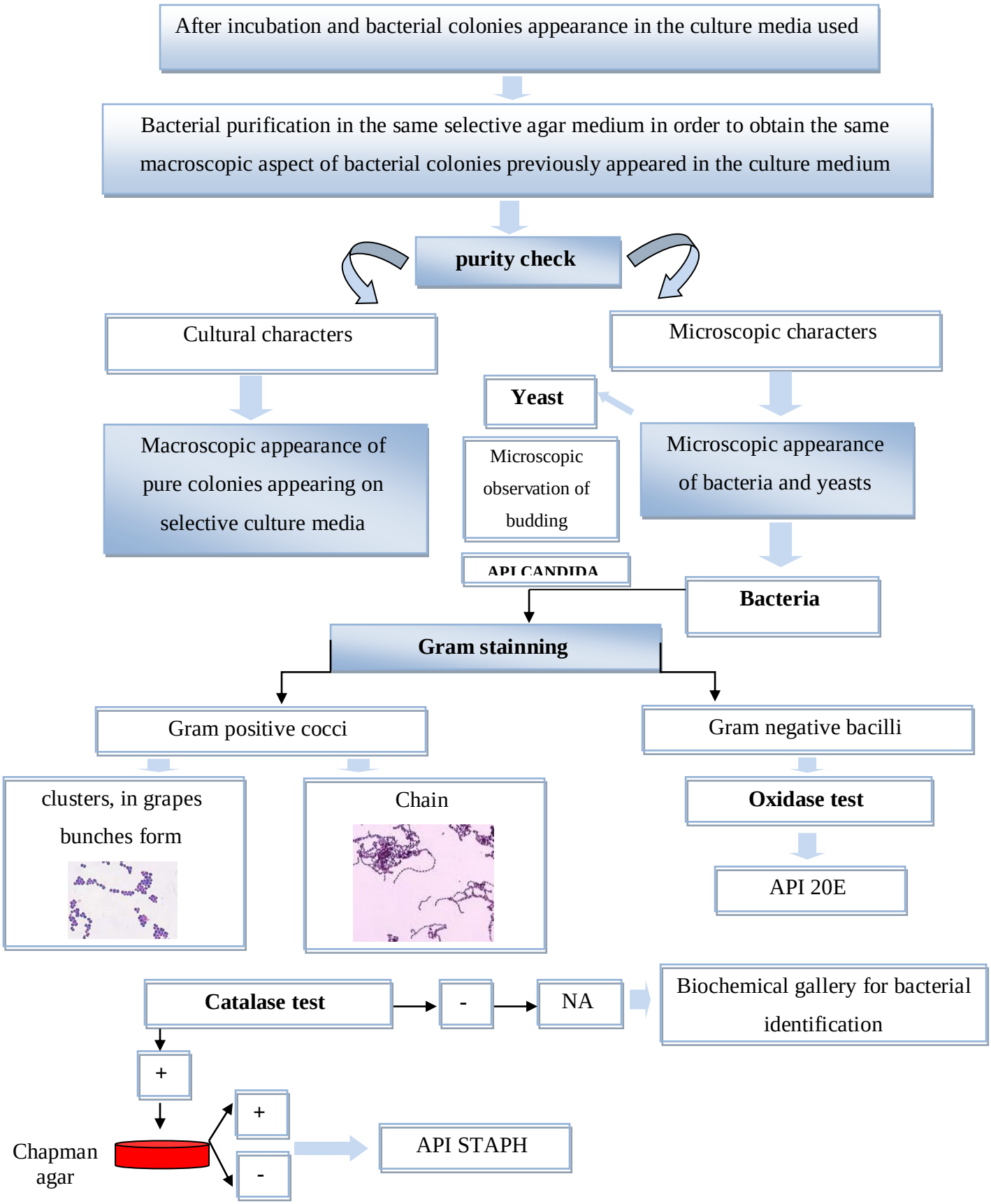


Figure 26: Summary diagram of the steps for identifying microbial strains.

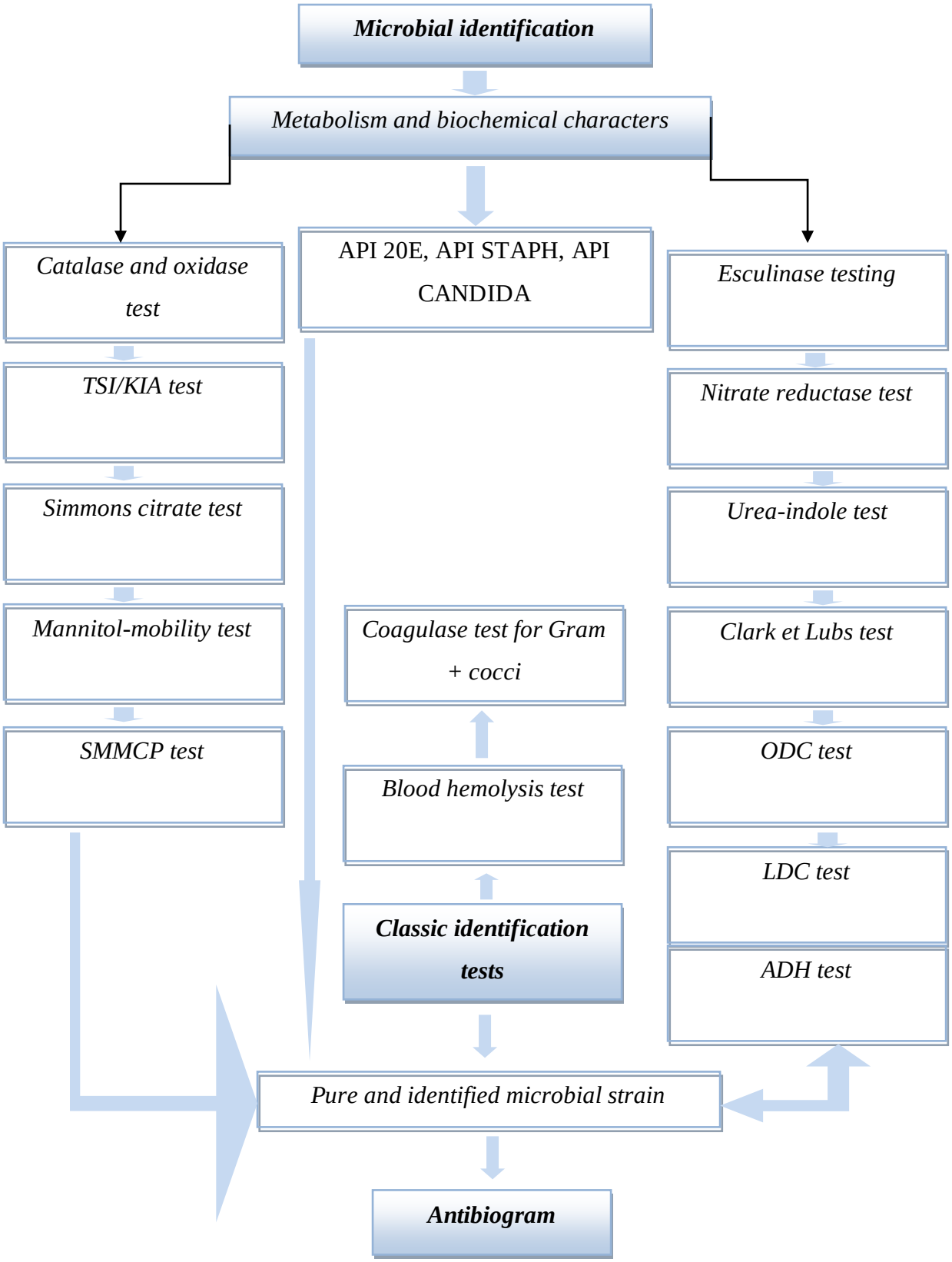


Figure 27: Microbial strains identification tests.

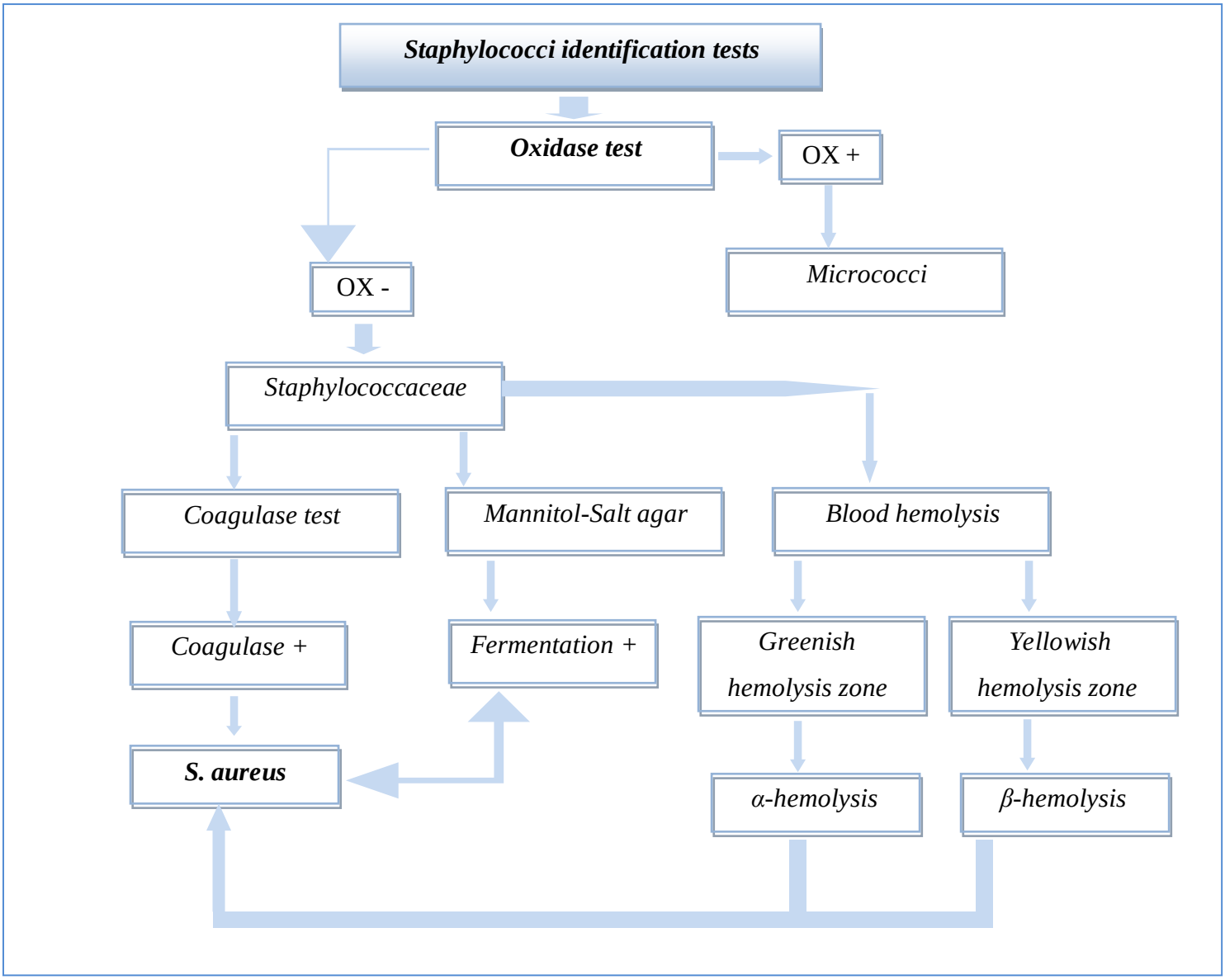


Figure 28: Key tests for staphylococci identification.

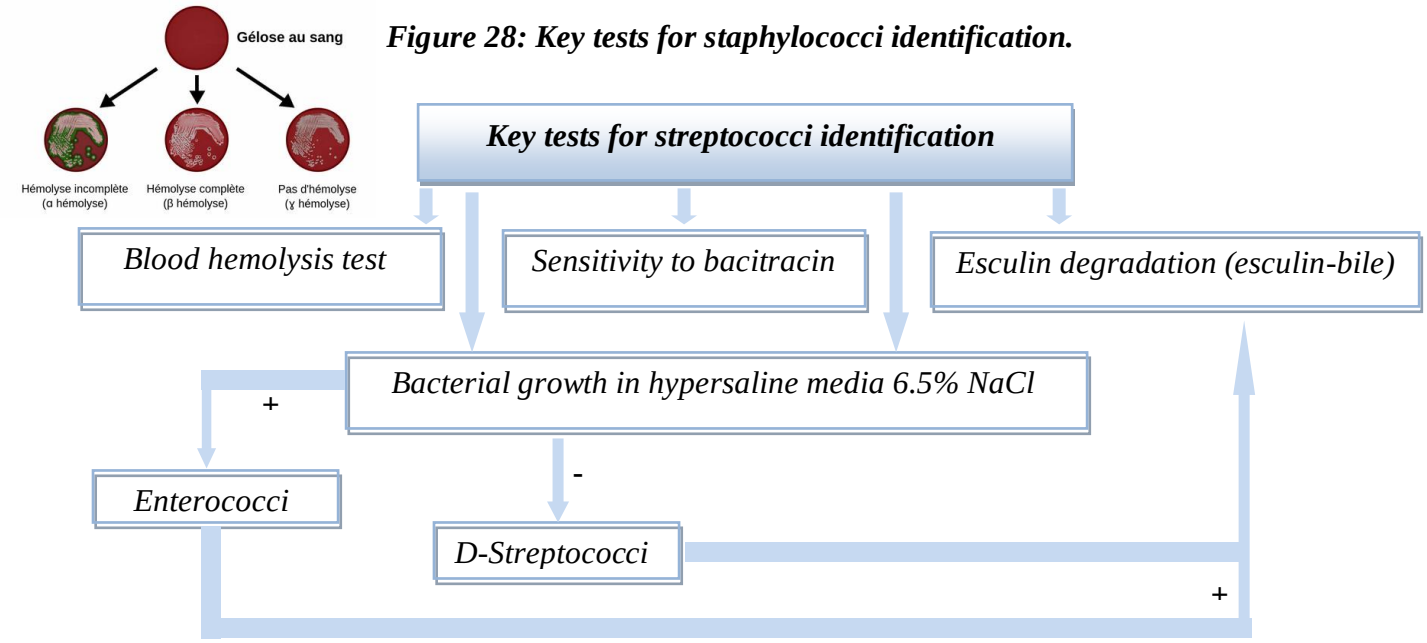


Figure 29: Key tests for streptococci identification.

II.3.1. Respiratory type study

- The inoculation is carried out on a meat-liver (MLA) agar previously regenerated in a boiling water bath for 20 minutes and when the temperature has dropped back down to 40-45 °C.
- This inoculation is carried out either using a Pasteur pipette from bottom to top in a spiral using the stab spiral inoculation method, or by depositing the studied bacteria suspension onto the tube surface maintained at 45 °C and then mixing, with the tube being held vertically, using 8-degree movements.
- The tubes are then cooled under tap water.
- After 24 hours at 37°C, the results lecture consists of studying the tube level where bacterial growth is visible (figure 30).

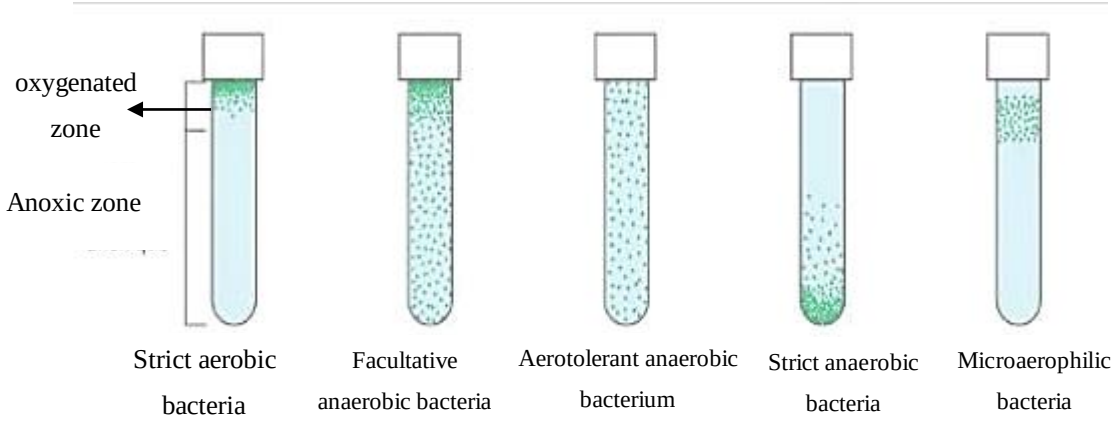


Figure 30: Study of bacterial respiratory type on meat-liver agar.

II.3.2. Catalase test

Two classic methods can be used to detect this enzyme:

Method 1:

- Take a hemolysis tube containing 1 mL of sterile physiological saline: - Emulsify a sufficient quantity of a 24-hour bacterial culture taken from agar medium, in order to obtain a thick suspension (high turbidity).
- Add 2 to 3 drops of 3% hydrogen peroxide without shaking.

- Wait 1 to 2 minutes before observing. The gas bubbles release indicates the presence of catalase.

Method 2:

- Take a clean blade for object preparation.
- Place a drop of 10 volume hydrogen peroxide on it and emulsify a little of the suspect colony or culture obtained on agar.
- Perform the test on the strains to be characterized and observe.

Results reading

The release of gas bubbles indicates the presence of catalase.

$$2H_2O_2 ==========> 2H_2O + O_2$$

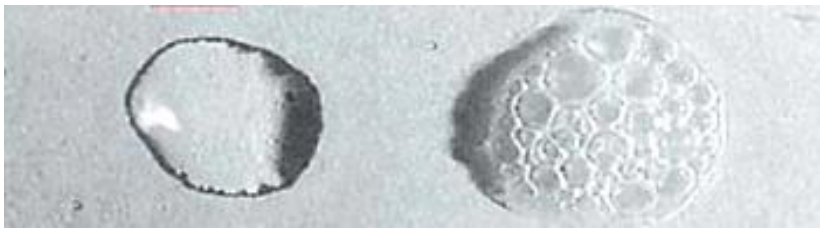
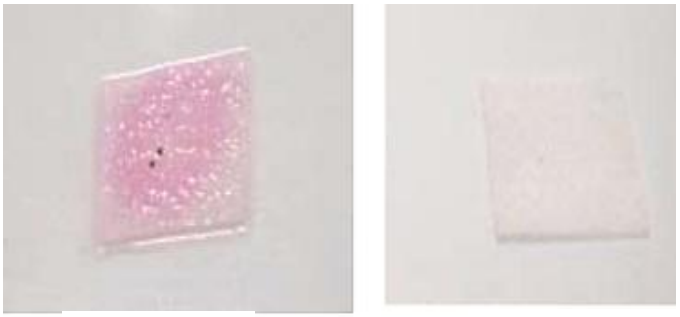


Figure 31: Catalase test results.

II.3.3. Oxidase test

This test makes it possible to highlight an enzyme: phenylenediamine oxidase of bacteria from their culture in agar medium. This enzyme is capable of oxidizing a reagent: N-Dimethyl-paraphenylenediamine. This reagent is colorless and in the presence of the enzyme, it releases a pinkish-red compound, which darkens in air (figure 32).



Oxidase + Oxidase -

Figure 32: Oxidase test results.

The reagent can be found in two forms:

- ✓ **Either in solution:** on a glass slide, place a square of filter paper and soak it with a freshly prepared reagent solution.

- ✓ **Either in the form of a pre-impregnated disc with the reagent:** In both cases, crush a bacterial colony on this paper with a Pasteur pipette tip (instrument does not oxidize the reagent). The platinum loop (nickel-chrome wire) should not be used as it can oxidize the reagent contained in the disc and give false positives.

II.3.4. Nitrate reductase test

When bacteria possess a nitrate reductase, they are able to transform nitrates (NO_3^-) into nitrites (NO_2^-) and possibly into nitrogen (N_2) (Figure 33). Nitrate reductase testing is performed on mannitol-mobility-nitrates agar or in conventional nitrate agar.

- A nitrate broth (nutritional broth supplemented with 1.5% potassium nitrates) is inoculated with the studied bacterium and incubated for 18 hours at 37°C (Figure 33A).

- After incubation, 3 drops of a sulfanilic acid solution (Griess A) and 3 drops of a naphthylamine solution (Griess B) are added to the bacterial culture.

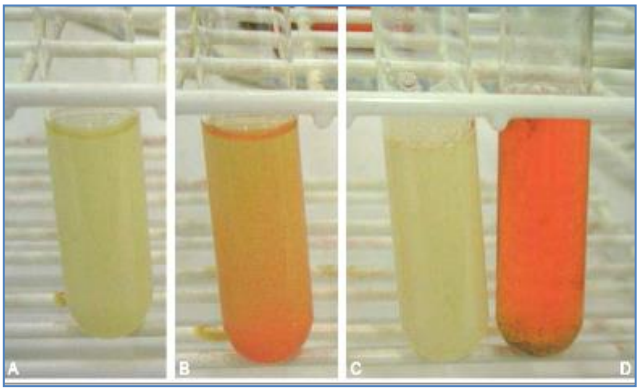


Figure 33: Nitrate reductase detection.

- If a fleeting pink color appears (Figure 33B), the nitrates have been reduced to the nitrite stage. In the absence of color, either the nitrates have been reduced to nitrogen, or the bacterium does not possess nitrate reductase. Adding zinc powder (Zobell's reagent, which reduces nitrates to nitrites) allows for a definitive solution. If a pink color appears, then

the bacterium does not possess nitrate reductase (Figure 33C). If no color change is visible after adding zinc, then the nitrates had been reduced to nitrogen (Figure 33D).

II.3.5. Carbohydrate Metabolism Study

II.3.5.1. SMMCP Test

- Bacteria can use carbohydrates via the fermentative pathway, which takes place in the absence of atmospheric oxygen and forms acidic catabolites, leading to a decrease in the environment pH, and the oxidative pathway, which uses atmospheric oxygen and few acidic catabolites are formed in this case.
- Two semi-solid media containing a pH indicator (e.g. bromothymol blue) are regenerated in a boiling water bath for 20 minutes.
- Then, when they have cooled to around 45°C, 6 drops of a 30% glucose solution (final glucose concentration of 1%) are added.
- The media are then cooled completely and inoculated using the stab inoculation method.
- One of the two tubes is coated with 0.5 cm of sterile paraffin oil. This tube constitutes the so-called "closed" tube, the tube in which the reactions will take place in the absence of oxygen.
- The other tube is called "open tube". The results of both carbohydrate attack pathways are presented in Figure 34.

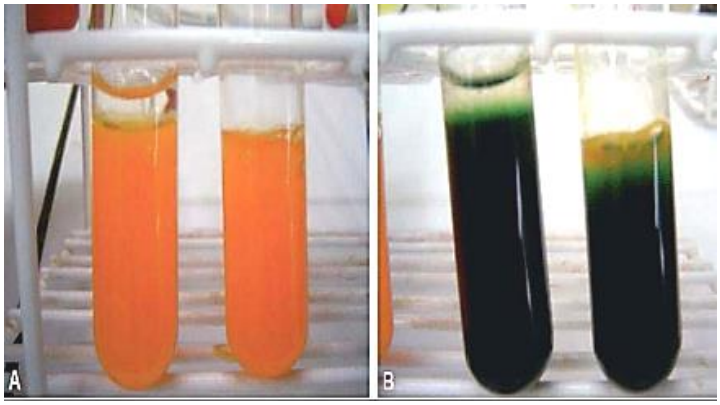


Figure 34: Study of the carbohydrate attack pathway.

II.3.5.2. Mannitol-Mobility test

- It is a semi-solid medium that contain mannitol and phenol red as a pH indicator in its composition.
- After regeneration in a boiling water bath for 20 minutes, the medium is completely cooled and then inoculated using the stab inoculation method.
- When the colored indicator changes from red to yellow, this color changes indicates that the mannitol has been used by the bacteria as carbohydrate and energy source, and which leads to medium acidification.
- The motile character is determined in this medium by a turbidity invading the entire agar width and also along the central stitch, whereas an immobile bacterium only develops along the central stitch.

II.3.5.3. Kligler-Hajna test

- Kligler-Hajna medium or lactose-glucose-H_2S medium is the most commonly used.
- This sloping solid medium contains glucose (0.1%), lactose (1%), amino acids, sodium thiosulfate, ferric citrate and phenol red.
- This medium is inoculated with the studied bacterial strain by making streaks on the agar slope surface, then the pellet is inoculated using the stab inoculation method.

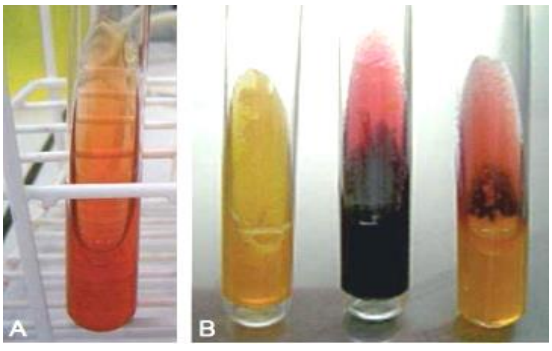


Figure 35: Kligler-Hajna results interpretation.

- Bacteria acidify glucose in anaerobic conditions inside the pellet (in the butt or bottom portion of Kligler-Hajna agar), which turns to a yellowish color after fermentation (for example, *Enterobacteriaceae*).

- If the germ does not use lactose, the slope turns red by re-alkalinization of the medium due to the alkaline products formation from the amino acid's degradation (e.g. *Proteus*) (Figure 35).

- If bacteria use lactose in relative aerobic conditions (slope), there is so a change in the slope towards yellowish color (e.g., *E. coli*).

- The medium can be colored black to varying degrees by the H_2S production. A fine spike of H_2S is observed for *Salmonella typhi*. The medium may be entirely black.

- The presence of gas is detected by the bubbles appearance or by agar lifting.

- Another medium containing 1% sucrose can be used: the TSI (Triple-Sugar-Iron) medium. All the experimental steps of bacterial inoculation are the same, except the TSI medium contains sucrose as 3rd carbohydrate source in addition to glucose and lactose.

II.3.5.4. ONPG test

- The ONPG test involves the detection of beta-galactosidase, which breaks down lactose into galactose and glucose.

- In practice, this test uses ortho-nitrophenyl-galacto-pyranoside (ONPG) or paranitrophenyl-galactopyranoside (PNPG).

- ONPG and PNPG, which diffuse spontaneously into the bacterium (unlike lactose, which requires a permease), are degraded by beta-galactosidase into galactose and orthonitrophenol or paranitrophenol respectively, which are yellow compounds.

- Orthonitrophenyl-galactopyranoside (ONPG) is a structural lactose analogue. This colorless synthetic substrate can be cleaved into galactose and orthonitrophenol (a yellow soluble compound) in the presence of ONPG hydrolase enzyme.

II.3.5.5. Clark et Lubs test

- Using Clark-Lubs's medium containing pyruvic acid, the formic acid and acetic acid formation (methyl red [MR] reaction), and the acetoin formation or acetyl-methylcarbinol (Voges-Proskauer [VP] reaction) are studied (Figure 36).

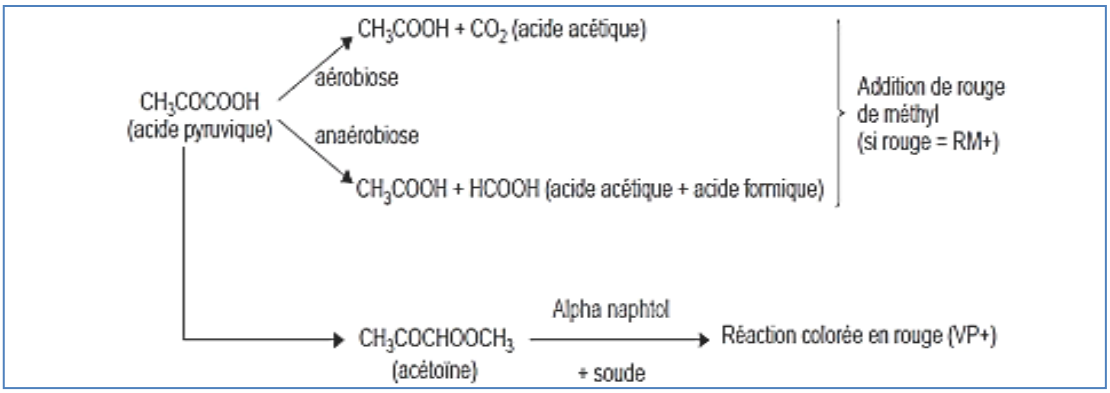


Figure 36: Highlighting the metabolites formed during the pyruvic acid degradation.

- In practice, the VP reaction is frequently performed, particularly on miniaturized galleries. Otherwise, Clark-Lubs's broth is inoculated and incubated for 18 hours at 37°C.
- To 1 ml of culture, add 0.5 ml of 6% α-naphthol and 0.5 ml of 16% sodium hydroxide.
- The results reading must be done within 10 minutes. *Enterobacteriaceae* of the genera *Klebsiella*, *Enterobacter* and *Serratia* produce acetoin, VP test is therefore +.

II.3.6. Protein metabolism study

II.3.6.1. Decarboxylases Detection

- Three decarboxylases are frequently studied: lysine decarboxylase (LDC), ornithine decarboxylase (ODC) and arginine dihydrolase (ADH).
- The corresponding tests are available in particular on the API 20 E® galleries.



Figure 37: Example of lysine decarboxylase detection in E. coli.

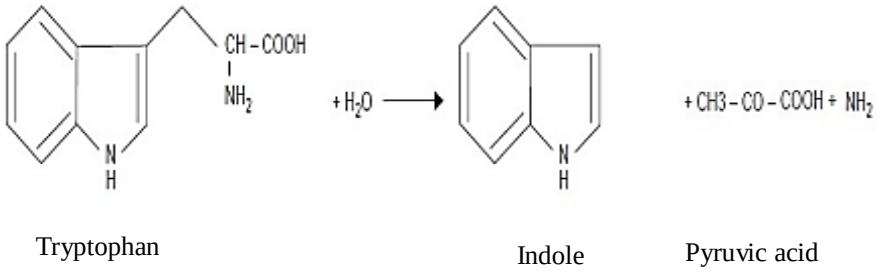
- The individual test can be performed on Taylor medium containing the amino acid being studied (lysine, ornithine or arginine), glucose and a colored indicator, bromocresol purple.

- The reaction is done on two stages. When glucose is fermented, there is a yellow turn of bromocresol purple, and when the amino acid is decarboxylated, there is a re-alkalinization of the medium which turns purple.

- After 18 hours at 37°C, a cloudy purple medium corresponds to a positive reaction (example: *E. coli* has an LDC while *Proteus* does not).

II.3.6.2. Tryptophanase testing

- The studied tryptophan is either directly supplied to the culture medium or contained in a high-tryptophan peptone medium which must be indole-free.



Tryptophan Indole Pyruvic acid

Figure 38: Demonstration of indole production.

- Through a complex reaction, tryptophan is broken down in the presence of tryptophanase into indole and other products.

- Indole production by tryptophan hydrolysis can be carried out from a 24-hour culture of the studied bacterial strain in Ferguson medium or in peptone water devoid of indole.

- The indole produced gives a red color in the presence of Kovacs' reagent (paradimethylaminobenzaldehyde + isoamyl alcohol) or James' reagent.

- The apperance of a red ring corresponds to a positive reaction, this is the case with *E. coli* and *P. mirabilis* which hydrolyzes tryptophan and produces indole.

II.3.7. Detection of desulfhydrases

- This research is carried out on Kligler-Hajna medium or is part of the gallery tests (API galleries).
- The sulfur amino acids degradation leads to the SH or H_2S formation which combine with the ammoniacal iron citrate present in the medium to form black iron sulfide.
- *Salmonella typhymurium* is H_2S positive while *E. coli* is H_2S negative.

II.3.8. Coagulase test

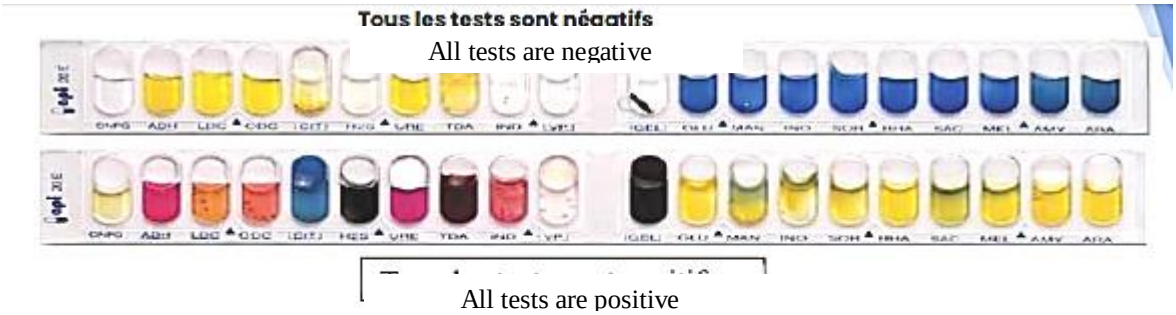
- Free coagulase is an exo-enzyme released into the external environment by *S. aureus*. Its identification confirms the presence of this bacteria .
- In a sterile test tube, 0.5 mL of oxalate serum is introduced with 0.5 mL of a bacterial suspension of *S. aureus* (Culture revived in BHIB).
- The mixture is kept in the incubator at a temperature of 37°C for 30 min and then observation of a coagulation reaction for up to 4h maximum (Kloss and Wolfshohl, 1982).

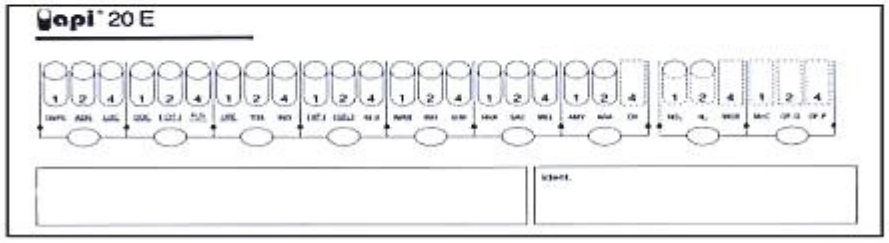
II.3.9. Blood hemolysis detection

- The search for blood hemolysin is carried out by isolating hemolytic bacteria on blood-enriched agar (*Streptococcus pyogenes*, *Staphylococcus* or β-hemolytic *E. coli*) with the demonstration of the different hemolysis types (Beutin et al., 1989).
- The blood-degrading bacteria present in the culture medium are surrounded by a hemolysis zone. This zone differs according to the hemolysin type: complete hemolysis for β-hemolytic bacteria, incomplete hemolysis for α-hemolytic bacteria, or an absence of hemolysis for non-hemolytic bacteria.

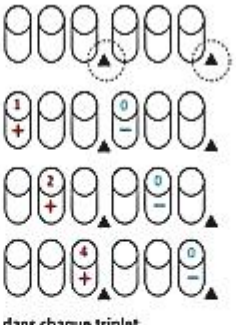
II.3.10 Reading results on API galleries

The following figures represent the reading results method on miniaturized API galleries (example of API 20 E).





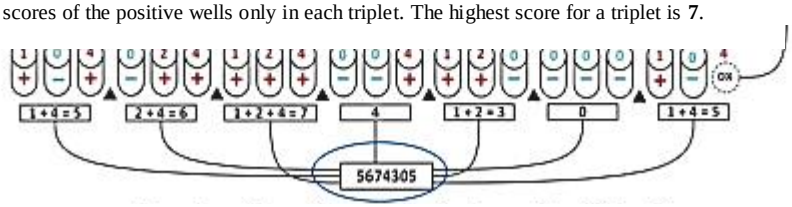
Results sheet of API 20E



1. The wells are delimited into triplets by **black triangles**.
2. For each triplet, if the first test is positive, the well will take **1**, if the test is negative, it will take 0.
3. For each triplet, if the second test is positive, the well will take **2**, if the test is negative, it will take 0.
4. For each triplet, if the third test is positive, the well will take **4**, if the test is negative, it will take 0.

Oxidase can also be included in the profile

5. Add together the scores of the positive wells only in each triplet. The highest score for a triplet is **7**.



Enter the digital profile into the API web to obtain the identity

Figure 39: Reading of API 20E gallery results for Enterobacteriaceae.

III. Antibiogram

The antibiogram almost always accompanies the pathogenic or potentially pathogenic bacteria identification. The antibiogram initiation must comply with the annual recommendations of the FMSAC (*French Microbiology Society of Antibiogram Comity*) on the antibiotics choice to be tested according to the involved bacteria. Performing an antibiogram is very important during bacterial identification. This test makes it possible to predict the sensitivity of a germ to one or more antibiotics, primarily for therapeutic purposes (Burnichon and Texie, 2003).

III.1. Standard antibiogram

- Antibiotic and antifungal discs are deposited on the Mueller Hinton agar surface previously inoculated with the pure and adjusted microbial suspension to 0.5 Mac Ferland (Burnichon and Texie, 2003). A final concentration of 10^6 or 10^8 cells/mL represented by an optical density of 0.08 to 0.1.
- Different antibiotics must be used in order to verify the antibiotic resistance profile of previously isolated and identified microbial strains.
- From the discs, the antibiotic diffuses into the MH agar, its concentration is lower when we move away from the disc center.
- After incubation at 37°C for 18 to 24 hours, each disc is surrounded by a microbial growth inhibition zone, the diameter of which varies depending on the antibiotic used.
- A small microbial growth inhibition zones diameters indicate that the tested microbial strain is resistant (Kubab *et al.*, 2006; Burnichon and Texie, 2003).
- The critical concentrations and diameters for each antibiotic mentioned by FSMAC., (2012) and FMS-AC/EUCAST, (2024) allow us to deduce whether a bacterial strain is sensitive or resistant to the chosen antibiotic.

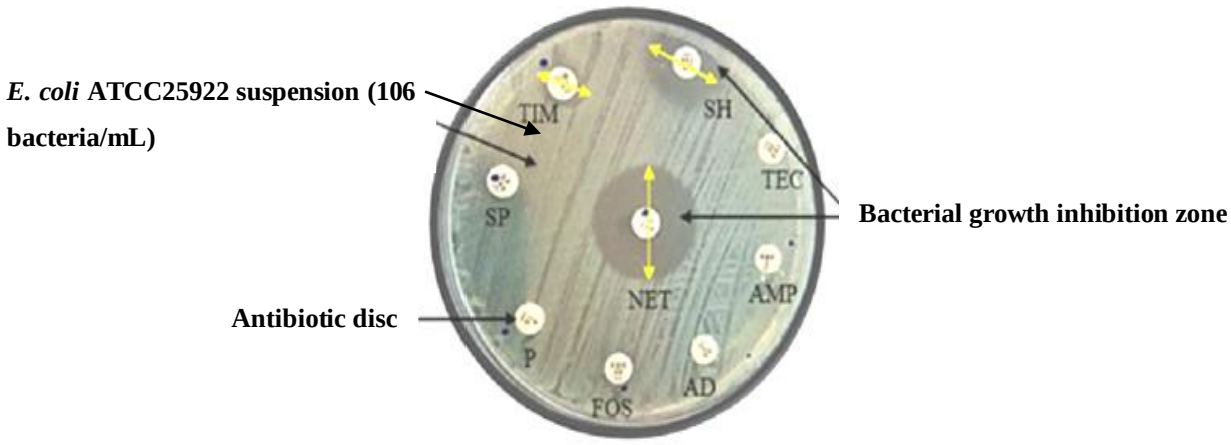
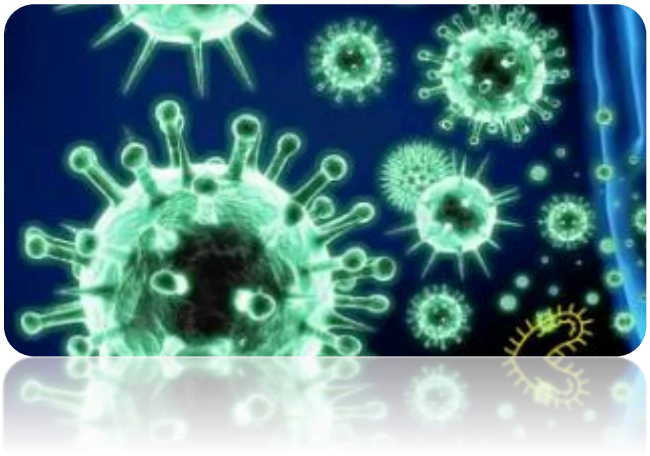


Figure 40: Antibiogram of a reference strain Escherichia coli ATCC 25922 to antibiotics.

Conclusion



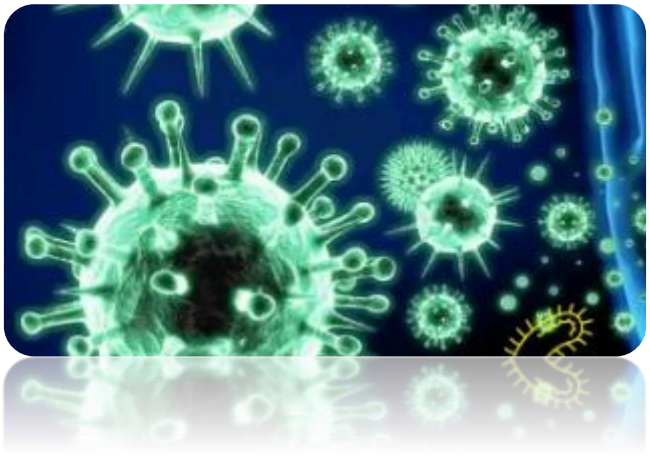
Conclusion

This practical handout has been designed to provide students with the fundamental theoretical knowledge and laboratory skills required for the study of general microbiology. Through the different practical sessions, students become familiar with laboratory biosafety rules, sterilization and disinfection techniques, culture media preparation, microbial inoculation methods, microscopic examination, bacterial identification, and antimicrobial susceptibility testing.

The successful application of these techniques requires accuracy, aseptic practices, critical observation, and strict compliance with laboratory safety guidelines. Mastery of these fundamental microbiological methods provides the basis for more advanced studies in medical, pharmaceutical, food, environmental, and industrial microbiology.

It is hoped that this handout will serve as a valuable learning resource, enabling students to develop both practical competence and scientific rigor while fostering good laboratory practices essential for their future academic and professional careers.

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