PW1: Gram-negative bacilli bacteria: Enterobacteria

1- Introduction

Members of the *Enterobacteriaceae* family, commonly termed enterobacteria, constitute a diverse group of Gram-negative, rod-shaped bacteria ubiquitous in nature and readily found in the digestive tracts of humans and animals. While many species reside harmlessly as commensals, others are significant pathogens implicated in gastrointestinal disease, urinary tract infections, bloodstream infections, and nosocomial outbreaks (Akmal et al., 2023).

Within this taxonomic group, certain genera warrant particular attention due to their clinical and ecological relevance. *Escherichia coli* is the most recognized and frequently studied species; it normally colonizes the gut without causing disease, yet pathogenic strains are major agents of diarrhea, urinary tract infections, and sepsis. In contrast, species such as *Salmonella enterica* (with its many serovars like *Typhi*, *Typhimurium*, *Enteritidis*) and *Salmonella bongori* act primarily as pathogens, responsible for gastroenteritis, systemic illnesses, and typhoid fever. The genus *Shigella*, which comprises *S. dysenteriae, S. flexneri, S. boydii*, and *S. sonnei*, is strictly pathogenic, being associated with bacillary dysentery; notably, *Shigella* species generally do not produce gas during glucose fermentation (Wang and Li, 2023).

Other genera in *Enterobacteriaceae* are more opportunistic, causing disease under favorable conditions. *Klebsiella pneumoniae* and *Klebsiella oxytoca* are encapsulated, nonmotile organisms frequently implicated in hospital-acquired pneumonia, septicemia, and urinary tract infections. Members of the genus *Enterobacter* (e.g. *E. cloacae*, *E. aerogenes*) are lactose-fermenting and often recovered from respiratory and urinary sites in nosocomial settings. *Proteus* species such as *P. mirabilis* and *P. vulgaris* are distinguished by their high motility and urease activity, traits that facilitate their role in urinary tract infections. Lastly, *Yersinia* includes *Y. pestis*, the agent of plague, as well as *Y. enterocolitica* and *Y. pseudotuberculosis*, which are associated with enteric disease (Wang and Li, 2023).

The distinguishing features among these taxa lie principally in their biochemical phenotypes, for instance, capacity to ferment lactose, motility, urease production, and gas formation during fermentation, as well as in their virulence potential. Lactose-fermenters like *E. coli* and *Klebsiella* contrast with non-fermenters such as *Salmonella* and *Shigella*. Motile species such as *Proteus* and many *Salmonella* strains differ from nonmotile genera like *Shigella* and

Klebsiella. These phenotypic distinctions are central to laboratory identification, and they reflect fundamental differences in ecology and pathogenicity.

A detailed understanding of biochemical and physiological traits is therefore vital for the correct classification, differentiation, and clinical management of enterobacteria. In this study, we adopt a combined laboratory approach, encompassing Gram staining, assays of respiratory type, nitrate reduction, catalase and oxidase activity, and carbohydrate profiling via API galleries, in order to delineate and interpret these critical microbial features (Anand and Griffiths, 2011).

2- Objectives

- ✓ Provide students with knowledge and practical skills to classify and differentiate strains of Enterobacteriaceae**.**
- ✓ Train students to apply standard microbiological and biochemical tests for accurate bacterial identification.
- ✓ Develop students' ability to interpret laboratory results effectively.

3- Techniques

For all tests, several fresh cultures of strains belonging to enterobacteria were previously prepared.

3-1- Gram staining

A fresh bacterial culture was used to prepare a thin smear on a glass slide, subsequently heat-fixed. The standard Gram staining procedure was followed: crystal violet (primary stain) was applied, followed by iodine solution (mordant), alcohol decolorization, and counterstaining with safranin. The slides were then examined under an oil-immersion objective (x10, x40 and x100). The bacteria appeared as pink rods, consistent with the Gram-negative morphology of enterobacteria.

Gram staining confirmed the expected cellular structure of enterobacteria. Their cell wall, characterized by a thin peptidoglycan layer and an outer membrane containing lipopolysaccharides, cannot retain crystal violet-iodine complexes during decolorization. This structural feature is of both taxonomic and clinical relevance, as it contributes to intrinsic antibiotic resistance and pathogenic mechanisms (Coico, 2006).

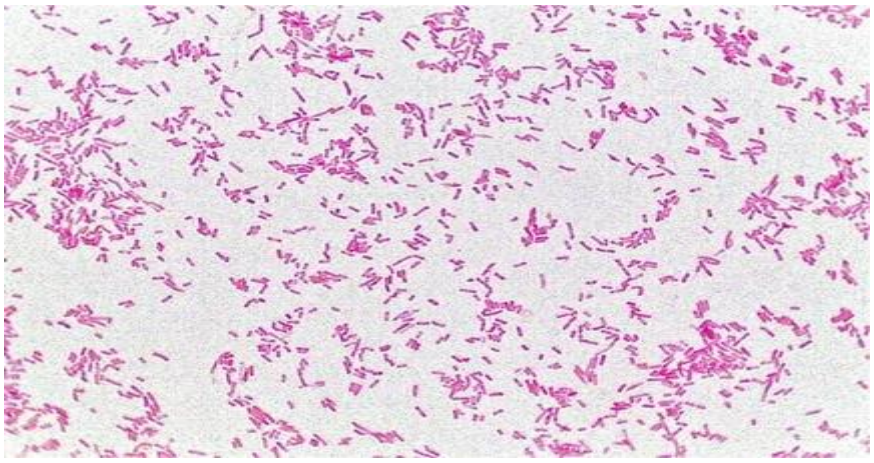

Figure 1. Gram staining of *E. coli* visualized at 100x magnification (Anwar et al., 2022)

3-2- Catalase test

The catalase test is used to detect the presence of the enzyme catalase, which catalyzes the breakdown of hydrogen peroxide (H_2O_2) into water and oxygen.

The procedure is straightforward: a small portion of a fresh bacterial colony is transferred onto a clean glass slide using a sterile loop. A drop of 3% hydrogen peroxide solution is then added directly onto the sample. If the organism produces catalase, rapid effervescence (bubbling due to oxygen release) will occur. This test should be performed on colonies grown on media without blood, as red blood cells contain catalase and may interfere, leading to false positive reactions (Taylor and Achanzar, 1972).

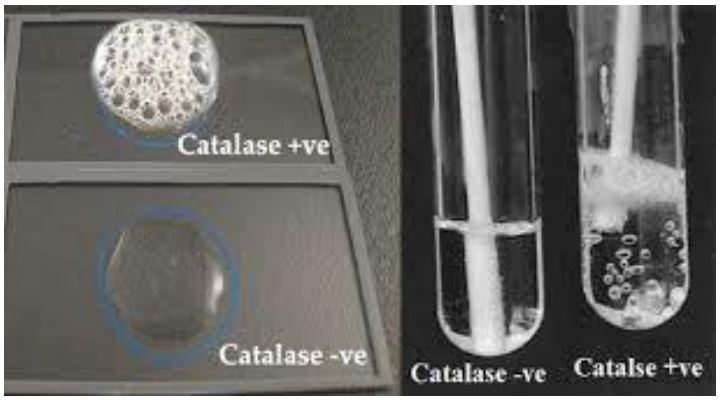

Figure 2. Catalase test. Positive for all Enterobacteriaceae (microbiologyinfo.com)

Results are interpreted as positive when immediate and abundant bubbles appear, while negative reactions show no visible effervescence. Members of the Enterobacteriaceae family are typically catalase positive, which helps distinguish them from Gram-positive cocci such as streptococci, which are catalase negative. Therefore, the catalase test is a valuable preliminary

tool in bacterial identification, as it quickly separates major groups and supports the classification of Gram-negative bacilli as likely belonging to catalase-producing families such as Enterobacteriaceae.

3-3- Oxidase test

The oxidase test detects the presence of the enzyme cytochrome c oxidase, a component of the bacterial electron transport chain.

The method involves placing a fresh bacterial colony onto a filter paper impregnated with a specific reagent, usually tetramethyl-p-phenylenediamine. In organisms possessing cytochrome oxidase, the reagent is rapidly oxidized and produces a violet to deep blue coloration within 30 seconds. It is crucial to interpret results promptly, as the reagent may undergo non-specific oxidation when exposed to air, leading to false positives if read too late.

A positive reaction is indicated by the rapid appearance of a violet coloration, whereas a negative reaction shows no significant color change. Enterobacteriaceae are characteristically oxidase negative, which provides an important criterion to distinguish them from other Gram-negative bacteria such as Pseudomonas or Neisseria, which are oxidase positive. Consequently, the oxidase test, together with Gram staining and catalase testing, forms part of the essential first-line assays in bacterial identification and classification (Shields and Cathcart, 2010).

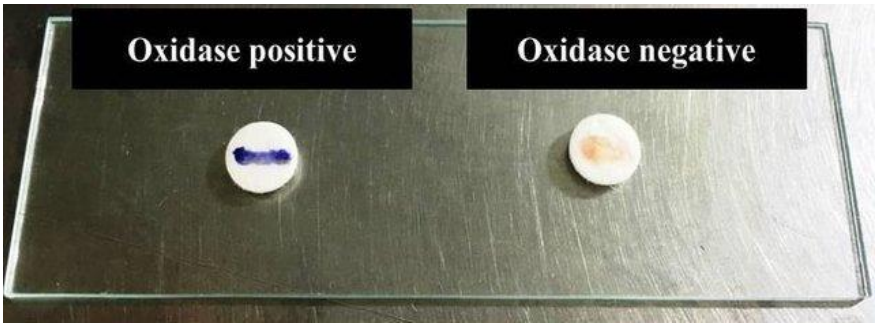

Figure 3. Oxidase test results (Sarangan et al., 2016)

3-4- Glucose/lactose catabolism

a. On Kligler Iron Agar (KIA)

- ✓ **Principe**

The KIA test is designed to study the carbohydrate metabolism of bacteria, especially within the Enterobacteriaceae family. Most enteric bacteria are able to ferment sugars, but they differ in their ability to ferment specific carbohydrates such as glucose and lactose, and in their ability

to produce gas or hydrogen sulfide (H_2S). These differences provide valuable criteria for bacterial identification.

KIA medium contains two sugars: glucose (0.1%) and lactose (1%). It also has peptones (for protein metabolism), phenol red as a pH indicator, and a system for detecting H_2S production (sodium thiosulfate + ferrous salts). The dual sugar system makes it possible to observe different fermentation patterns: glucose only, glucose + lactose, or no sugar fermentation. In addition, the medium allows the observation of gas production and H_2S formation (Skillern and Overman, 1983).

- **Glucose** is the simplest carbohydrate and is usually fermented by most Enterobacteriaceae. Fermentation produces acidic end-products, which lower the pH and cause a visible color change in the medium due to the pH indicator. Because glucose is present in a small amount in KIA, only temporary acidification of the slant occurs; later, bacteria switch to peptone catabolism under aerobic conditions, releasing alkaline compounds.
- **Lactose** being a disaccharide, requires the presence of β-galactosidase for utilization. Only some enteric bacteria can ferment lactose. When lactose is fermented, the amount of acid produced is greater and more stable, keeping both the slant and butt acidic (yellow). This ability to ferment lactose is one of the key points for distinguishing bacteria such as *E. coli* (lactose fermenter) from *Salmonella* or *Shigella* (non-lactose fermenters).

✓ **Inoculation**

For inoculation of Kligler Iron Agar, a sterile straight inoculating needle should be used rather than a loop, since the medium must be penetrated without causing unnecessary damage.

The procedure begins with a deep stab into the butt of the agar by inserting the needle straight down to the bottom of the tube. This step allows bacterial growth in anaerobic conditions, making it possible to observe glucose fermentation and hydrogen sulfide (H_2S) production. After withdrawing the needle, the surface of the slant is then streaked in a zig-zag pattern, providing an aerobic environment where lactose fermentation or peptone utilization can be detected. This combined stab-and-streak inoculation technique is essential for interpreting the different metabolic reactions that occur in both the aerobic and anaerobic zones of the medium (Taylor and Silliker, 1958).

✓ **Results interpretation**

After 24 to 48 h of incubation:

- Glucose fermentation only → butt yellow, slant red.
- Glucose + lactose fermentation → butt and slant yellow.
- Gas production → cracks, bubbles, or agar displacement.
- H_2S production → black precipitate in the butt.

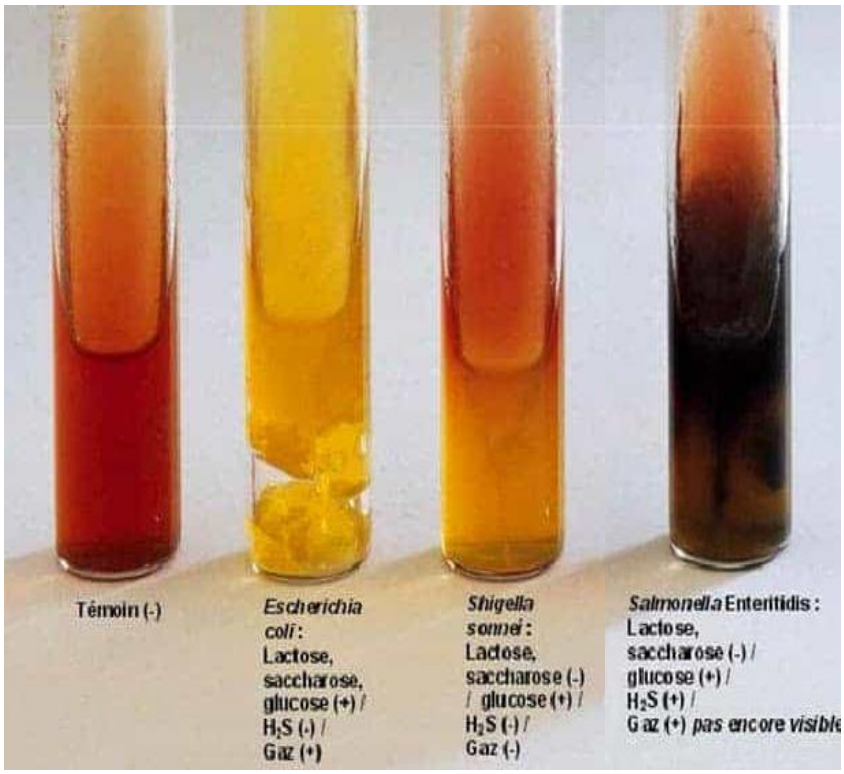

Figure 4. Aspects of some Enterobacteriaceae species on KIA medium (microbiologyinfo.com)

b. On MacConkey Agar and EMB Agar

✓ **Principe**

MacConkey agar and Eosin Methylene Blue (EMB) agar are both selective and differential culture media designed for the isolation of Gram-negative enteric bacteria. Their selectivity comes from the presence of bile salts and dyes, which inhibit the growth of most Gram-positive organisms, allowing primarily Gram-negative bacilli to develop. They are also differential because they contain lactose as a fermentable carbohydrate, and an indicator system that distinguishes lactose fermenters from non-fermenters (Allen, 2005).

On MacConkey agar, bacteria that ferment lactose produce acid, lowering the pH and causing colonies to appear pink to red. Non-lactose fermenters, such as *Salmonella*, *Shigella*, or *Proteus*, do not produce acid and therefore form pale or colorless colonies.

On EMB agar, the same principle applies, but with an additional visual effect: strong lactose fermenters like *Escherichia coli* produce abundant acid that reacts with the dyes (eosin and methylene blue), resulting in characteristic metallic green sheen colonies. Weaker lactose fermenters (*Enterobacter*, *Klebsiella*) appear as pink to purple colonies, while non-lactose fermenters form colorless or translucent colonies (Divya et al., 2016).

- ✓ **Inoculation**

Inoculation is done using a sterile loop with the streak plate method on the surface of the agar. The plate is then incubated at 37 °C for 18–24 h.

- ✓ **Results interpretation**

These conditions allow the growth of Gram-negative bacteria and differentiation between lactose fermenters.

MacConkey Agar

- Lactose fermenters → pink to red colonies (e.g., *E. coli*, *Klebsiella*).
- Non-lactose fermenters → colorless or pale colonies (e.g., *Salmonella*, *Shigella*).

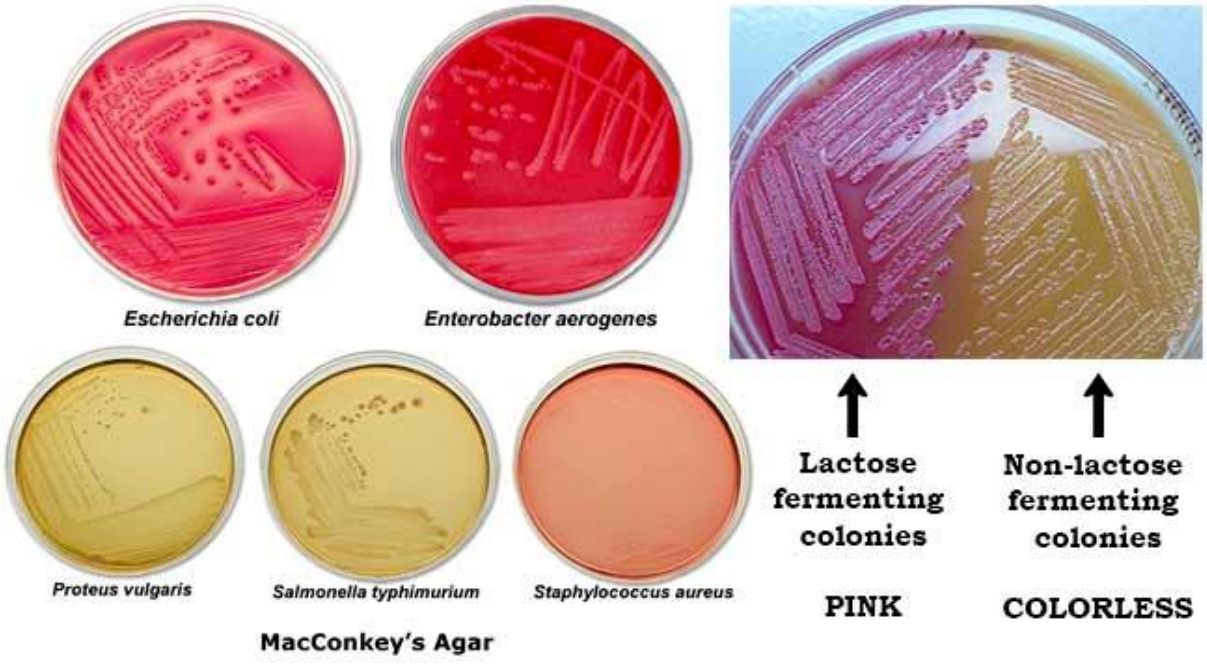

Figure 5. Appearance of different species on MacConkey's agar (microbiologyinfo.com)

EMB Agar

- Strong lactose fermenters → metallic green sheen colonies (typical of *E. coli*).
- Weak lactose fermenters → pink to purple colonies (e.g., *Enterobacter*).
- Non-lactose fermenters → colorless or translucent colonies (e.g., *Salmonella*, *Shigella*).

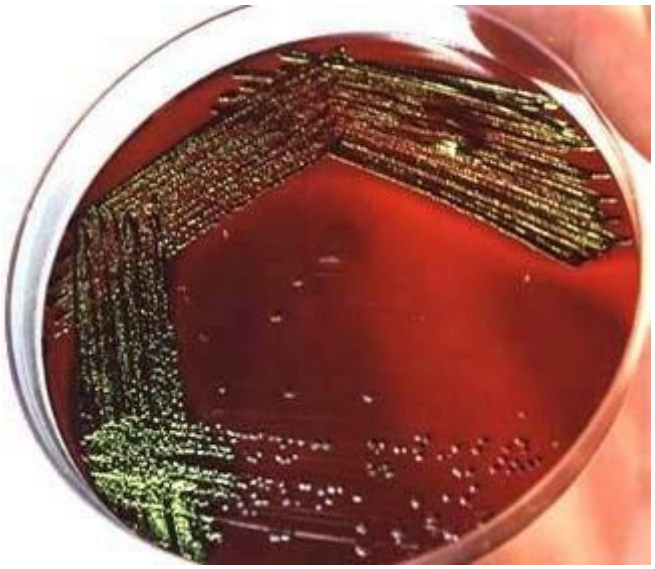

Figure 6. Appearance of *E. coli* on EMB agar with metallic sheen (Divya et al., 2016)

3-5- Mannitol catabolism and mobility

The mannitol fermentation test is useful, as it reveals whether bacteria can metabolize mannitol to produce acid (and sometimes gas). Many Enterobacteriaceae ferment mannitol, but some pathogenic strains, such as certain *Shigella* species, do not. This difference provides valuable diagnostic information and supports the classification of isolates during laboratory identification.

The motility test is an important tool in microbiology because it helps distinguish between genera and species of Enterobacteriaceae based on their ability to move using flagella. For example, E. coli and Salmonella are motile, while *Klebsiella* and *Shigella* are non-motile. This simple test therefore plays a key role in bacterial differentiation and identification (Shields and Tsang, 2006).

- ✓ **Principe**

Mannitol Motility Nitrate Agar is a polyvalent differential medium used to study three important bacterial characteristics in a single tube: (1) the ability to ferment mannitol with acid production, (2) the capacity for motility due to flagella, and (3) the potential to reduce nitrate

(NO_3^-) into nitrite (NO_2^-) or further end-products. These parameters are particularly useful in the identification and differentiation of Enterobacteriaceae (Shields and Tsang, 2006).

- ✓ **Composition**

Mannitol Motility Nitrate Agar contains several ingredients, each serving a specific role in the study of bacterial physiology. Mannitol is provided as the main carbohydrate to test fermentation; if bacteria can metabolize it, acid is produced and the pH of the medium decreases. Peptone supplies nitrogen and other essential nutrients to support bacterial growth, even for organisms that do not ferment mannitol. To visualize changes in pH, the medium includes phenol red, a pH indicator that turns yellow when acid is formed, helping to identify positive fermentation. The agar concentration is kept low (0.3–0.4%), creating a semisolid consistency that allows motile organisms to diffuse away from the stab line, making it possible to detect bacterial motility. In addition, the medium contains potassium nitrate (KNO_3), which acts as the substrate for testing nitrate reduction through bacterial nitrate reductase activity. In some preparations, a Durham tube may be inserted to capture gas bubbles, allowing the detection of nitrogen gas released during nitrate reduction (Shields and Tsang, 2006).

- ✓ **Inoculation**

The medium is inoculated by inserting a sterile straight needle with the bacterial culture in a single stab down the center of the tube. This technique allows simultaneous observation of fermentation in the butt, motility in the agar depth, and nitrate reduction. Tubes are incubated at 37 °C for 24–48 h. After incubation, specific reagents (sulfanilic acid and α-naphthylamine, and possibly zinc powder) are added to detect nitrate reduction.

- ✓ **Results and interpretation**

Mannitol fermentation:

Positive → medium turns yellow (acid production).

Negative → medium remains red or orange (no fermentation).

Motility:

Positive → diffuse, cloudy growth spreading away from the stab line.

Negative → growth limited to the stab line only.

Nitrate reduction:

Positive ($NO_3^- \rightarrow NO_2^-$) → red color after addition of reagents.

Positive (NO_3^- → N_2 gas) → gas in Durham tube or no color change but confirmed by absence of red after zinc.

Negative → red color only after adding zinc (indicating unreduced nitrate still present).

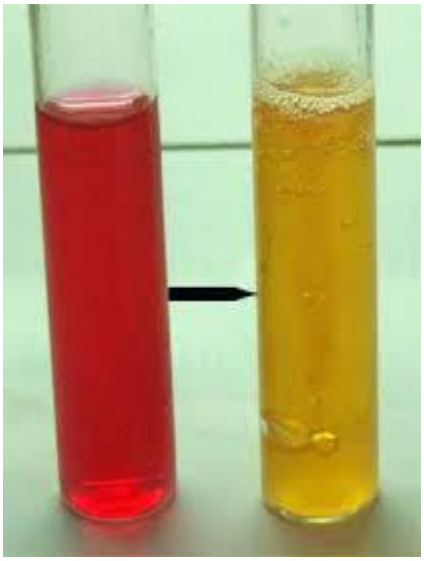

Figure 7. Mannitol mobility medium, negative (left) and positive (right)

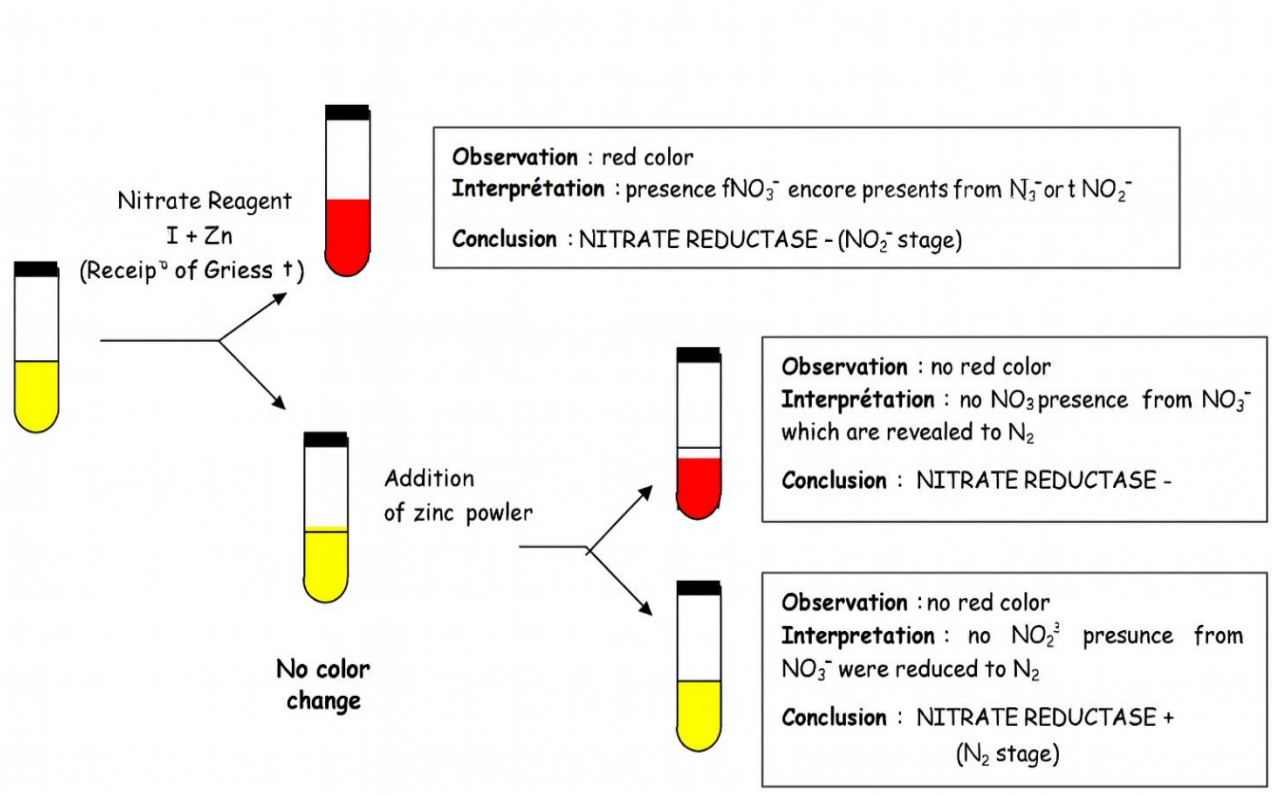

Figure 8. Explanatory scheme of the nitrate reductase detection procedure after specie incubation on Mannitol Mobility Agar

3-6- API® 20E Gallery

The API 20E is a standardized, miniaturized identification system designed for the differentiation of Enterobacteriaceae and other Gram-negative rods. It is widely used in clinical and food microbiology because it allows a large number of biochemical tests to be performed simultaneously, leading to rapid and reliable bacterial identification (Holmes et al., 1978).

a. Principle

The gallery consists of 20 microtubes containing dehydrated substrates. When inoculated with a bacterial suspension, metabolic activities such as carbohydrate fermentation, enzyme production, or substrate utilization produce visible color changes. Each reaction is recorded as positive or negative, generating a numerical profile code, which is then interpreted using the API database to identify the species (Holmes et al., 1978).

b. Composition / contents of the strip (20 tests)

- Sugar fermentation tests**:** glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin, arabinose.
- Enzyme activity tests: β-galactosidase (ONPG), lysine decarboxylase (LDC), ornithine decarboxylase (ODC), urease, tryptophan deaminase (TDA), gelatinase (GEL).
- Respiration tests**:** citrate utilization (CIT), H_2S production, oxidase.
- Indole production (IND) **and** acetoin production (VP, Voges–Proskauer) (bioMérieux, France).

c. Inoculation

A bacterial suspension (usually adjusted to a 0.5 McFarland standard) is prepared from a pure, fresh culture. This suspension is used to inoculate each microtube of the strip. Some tubes are overlaid with mineral oil (to create anaerobic conditions), while others remain open. The strip is placed in the incubation tray with a small volume of water to maintain humidity and incubated at 37 °C for 18-24 h (bioMérieux, France).

d. Results interpretation

- After incubation, the reactions are read based on color changes. Some tests require the addition of reagents (e.g., Kovac's reagent for indole, ferric chloride for TDA, VP reagents for acetoin). Positive and negative reactions are grouped into sets of three, and each set is given a numerical value (1, 2, or 4). Adding these values produces a seven-digit profile

number, which is then checked in the API 20E identification database or software to determine the bacterial species (bioMérieux, France).

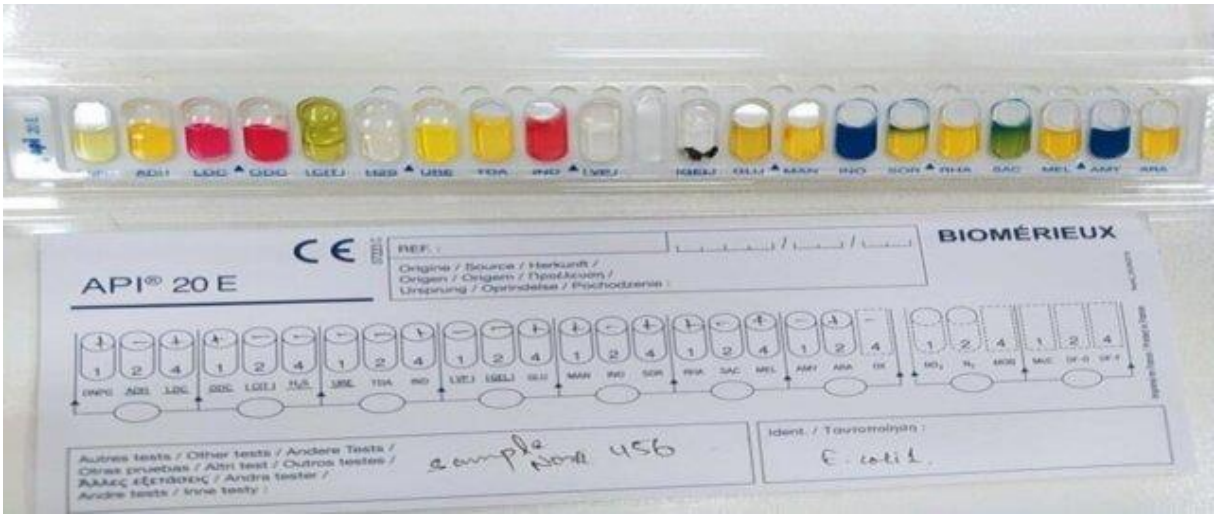

Figure 9. API 20E results for *E. coli* (Akmal et al., 2023).

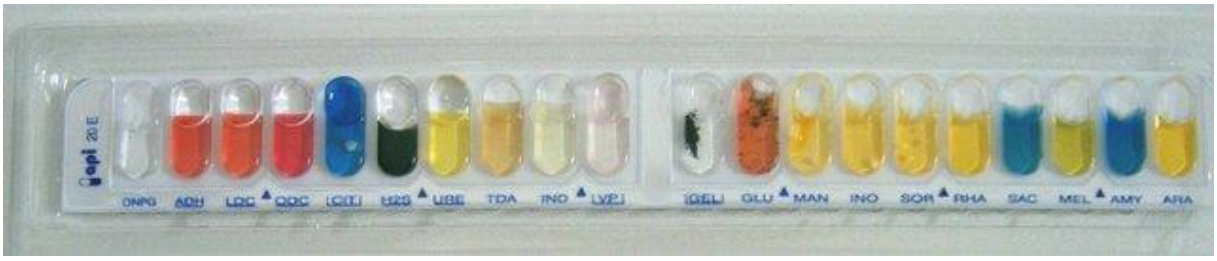

Figure 10. Typical *Salmonella* reaction of API 20E test kit (Ben Salem et al., 2012).