PW4: Gram-positive cocci bacteria

Lactic acid bacteria: *Lactococcus* sp and *Enterococcus* sp

1- Introduction

Gram-positive cocci represent a significant group of bacteria with wide ecological, industrial, and clinical importance. They are characterized by a thick peptidoglycan cell wall, which retains the crystal violet stain in the Gram staining method, giving them their distinctive purple color under the microscope. Among this group, some species are pathogenic (such as *Staphylococcus* and *Streptococcus*), while others, particularly the lactic acid bacteria (LAB), are considered beneficial or play dual roles depending on the context (König and Fröhlich, 2017).

LAB are defined by their ability to ferment carbohydrates into lactic acid as the major metabolic end product. Within this group, *Lactococcus* spp. and *Enterococcus* spp. are of special interest. *Lactococcus* is mainly associated with the dairy industry, where it acts as a starter culture in cheese and fermented milk production, contributing to acidification, texture formation, and flavor development. In contrast, *Enterococcus* spp. illustrate the dual nature of LAB: while certain strains participate in traditional food fermentations, others are well-known as opportunistic pathogens in humans, responsible for various clinical infections and noted for their antibiotic resistance (Khalid, 2011).

2- Objectives

- ✓ Perform and interpret key differential tests (Gram staining, catalase, growth at different temperatures, salt tolerance, fermentation profiles, etc.) to distinguish the two homofermentative genera *Lactococcus* from *Enterococcus*.
- ✓ Understand the ecological and industrial significance of LAB.

3- Techniques

3-1- Gram staining (non-differential test)

This differential staining technique separates bacteria based on their cell wall. Both *Lactococcus* and *Enterococcus* appear as Gram-positive cocci, purple in color, usually in pairs or chains. Since this method was already detailed in previous practical works, here it serves mainly as a confirmation step before biochemical tests.

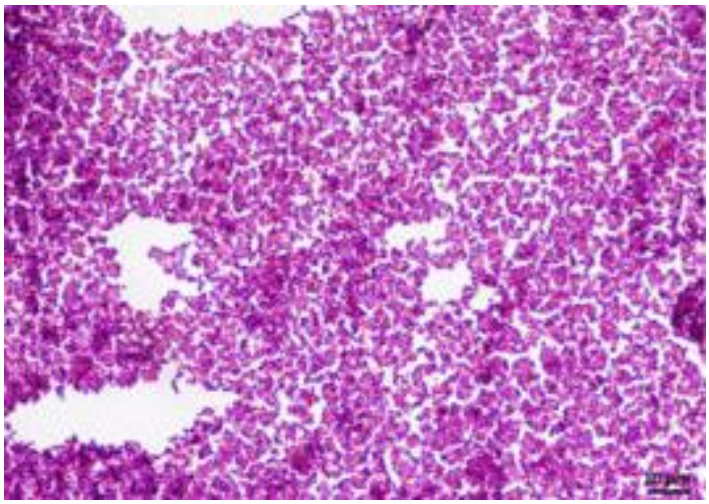

Figure 23. Gram staining of *Lactococcus lactis* (Maślak et al., 2022)

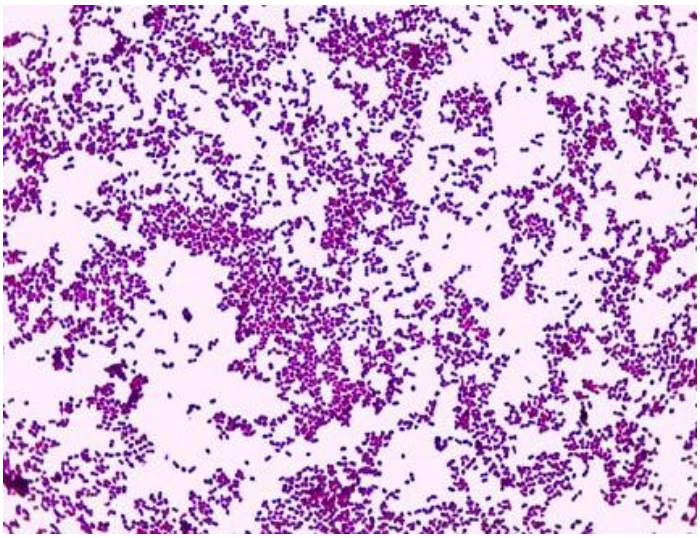

Figure 24. Gram staining of *Enterococcus* sp (Fayed, 2012)

3-2- Catalase test (non-differential test)

This test detects the enzyme catalase, which breaks down hydrogen peroxide into water and oxygen (visible as bubbles). Both *Lactococcus* and *Enterococcus* are catalase-negative, which distinguishes them from staphylococci (catalase-positive). The method was already explained in detail in PW2, so here it is used mainly as a confirmatory test for Gram-positive cocci LAB (Ammor et al., 2005).

3-3- Fermentative type (non-differential test)

The test is used to determine whether a lactic acid bacterium follows a homofermentative or heterofermentative pathway (Ammor et al., 2005).

a. Principle

MRS-G broth (MRS medium where lactose is replaced by glucose) is used as a fermentation medium. A Durham tube is placed inside to trap any CO_2 released during fermentation.

- If the organism ferments glucose heterofermentatively, CO_2 accumulates in the Durham tube as a visible gas bubble.
- Homofermenters do not produce gas, so the Durham tube remains filled with liquid.

b. Inoculation

A sterile inoculating loop is used to inoculate a fresh culture of the test strain into the MRS-G broth containing a Durham tube. Tubes are incubated at 30 °C for 24 h under appropriate conditions.

c. Results interpretation

- **Positive (heterofermentative metabolism):** Presence of a visible gas bubble in the Durham tube, showing CO_2 production from glucose fermentation (e.g., *Leuconostoc* spp.).
- **Negative (homofermentative metabolism):** No gas bubble in the Durham tube, only lactic acid is produced (e.g., *Lactococcus lactis*).

Figure 25. Fermentative type of homofermentative LAB (A) and heterofermentative LAB (B)

3-4- Arginine hydrolysis test (non-differential test)

The arginine hydrolysis test is used to detect the presence of the enzyme arginine dihydrolase (ADH) in LAB. This enzymatic activity allows certain strains to metabolize arginine, differentiating them from other LAB that ferment sugars only (Moraes et al., 2013).

a. Principle

The test is performed in M16 BCP medium, which contains lactose as a fermentable sugar, arginine as the specific substrate, and bromocresol purple as a pH indicator. Lactic acid bacteria ferment lactose, producing acid and lowering the pH, which causes the colonies and surrounding medium to turn yellow. Strains that can hydrolyze arginine generate alkaline products during metabolism, which realkalinize the medium and restore the indicator's original violet color.

b. Composition of the medium

The M16 BCP medium is composed of lactose (2 mg/ml) as the carbohydrate source, arginine (4 mg/ml) as the substrate for ADH, and bromocresol purple (0.05 mg/ml) as a pH indicator. The base medium (M16) also provides peptones, yeast extract, and mineral salts to support bacterial growth.

c. Inoculation

A pure culture of the strain under study is inoculated into M16 BCP medium and incubated at 30 °C for 24-48 h under appropriate growth conditions for LAB.

d. Results interpretation

- **Positive (ADH+):** The medium first turns yellow due to lactose fermentation, then reverts to violet as arginine is hydrolyzed and alkaline by-products are released.
- **Negative (ADH–):** The medium remains yellow after fermentation, with no re-alkalinization, indicating the absence of arginine dihydrolase activity.

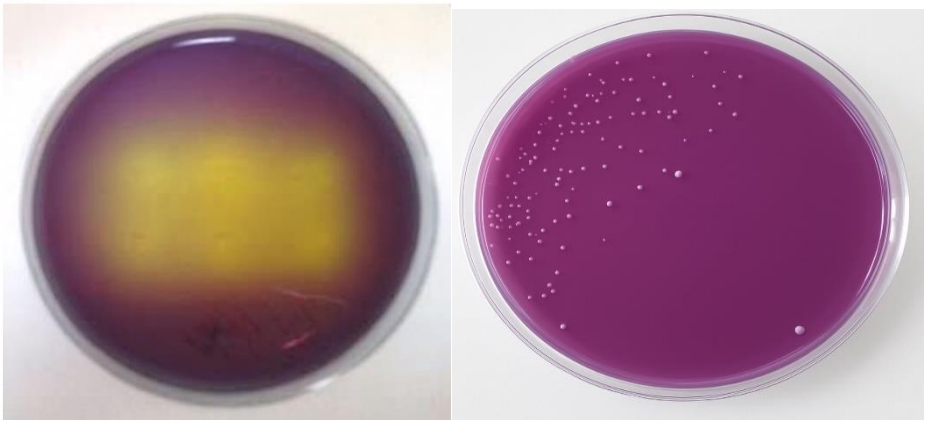

Figure 26. Arginine hydrolysis detected on M16BCP, yellow: enzyme absence (left); Purple: enzyme presence (right) (Zarour, 2010)

3-5- Growth at different temperatures (differential test)

Temperature tolerance is an important physiological criterion to distinguish *Lactococcus* from *Enterococcus*. LAB such as *Lactococcus* are adapted mainly to cooler, nutrient-rich environments (like milk), whereas *Enterococcus* spp. are more resilient and able to grow under harsher conditions, including higher temperatures (Moraes et al., 2013).

a. Principle

The test is based on the ability of bacteria to grow in nutrient-rich broth incubated at specific temperatures. *Lactococcus* spp. typically grow at 10 °C, but do not grow at 45 °C, reflecting their adaptation to dairy environments. *Enterococcus* spp., in contrast, can grow at both 10 °C and 45 °C, showing their wide ecological tolerance.

b. Composition of the medium

A simple nutrient broth or MRS broth (containing peptones, yeast extract, glucose, and salts) is sufficient. The medium provides the basic nutrients required for LAB growth, while temperature is the key selective factor.

c. Inoculation

Tubes of broth are inoculated with a pure culture from a fresh colony using a sterile loop. At least two tubes are prepared for each strain: one incubated at 10 °C and the other at 45 °C, usually for 24-48 h.

d. Results and interpretation

- *Lactococcus* spp. → Growth at 10 °C (turbidity), no growth at 45 °C (clear broth).
- *Enterococcus* spp. → Visible growth at both 10 °C and 45 °C (turbidity in both tubes).
- This clear difference helps in distinguishing between the two genera.

3-6- Growth in salt (NaCl tolerance test) (differential test)

Tolerance to high salt concentration is an important physiological trait to differentiate *Lactococcus* from *Enterococcus*. Salt inhibits the growth of many LAB, but *Enterococcus* spp. are adapted to harsher conditions and can grow in the presence of 6.5% NaCl, which is a key diagnostic criterion (Thapa et al., 2006).

a. Principle

The test evaluates the ability of bacteria to grow in a broth medium containing 6.5% NaCl. High osmotic pressure restricts the growth of most lactic acid bacteria, but enterococci can survive and multiply under these stressful conditions, making this a reliable test to distinguish them.

b. Composition of the medium

A basic nutrient broth or MRS broth supplemented with 3 and 6.5% NaCl is used.

- Peptones and yeast extract → provide nitrogen and growth factors.
- Glucose → fermentable carbohydrate.
- NaCl (3 and 6.5%) → selective factor to test salt tolerance.

c. Inoculation

Tubes of the broth with 3 and 6.5% NaCl are inoculated with a pure bacterial colony using a sterile loop. The tubes are incubated at 37 °C for 24-48 h.

d. Results and interpretation:

- *Lactococcus* spp. → No growth or very poor growth (broth remains clear).
- *Enterococcus* spp. → Good growth, visible turbidity in the broth.

3-7- Sherman's blue test (differential test)

a. Principle

This test evaluates the ability of bacteria to reduce methylene blue (Sherman's blue) in milk. The reduction is linked to bacterial metabolism and oxygen consumption. Rapid decolorization indicates strong reducing activity, which is typical of certain enterococci (Kirschner et al., 2001).

b. Procedure

Sterile milk supplemented with methylene blue (to final concentration of 0.01%) is inoculated with the bacterial strain.

The tube is incubated at 37 °C. The time taken for the blue color to disappear (decolorization) is recorded (from 30 min to 24h).

c. Results and interpretation

Lactococcus spp → generally show slower reduction, with the blue color persisting longer.

Enterococcus spp → rapid reduction of methylene blue, leading to quick decolorization (within a few hours).

This test is mainly used in dairy microbiology to differentiate between true lactic streptococci (Lactococcus), which are essential starter cultures, and enterococci, which may be contaminants or indicators of hygiene quality in milk.

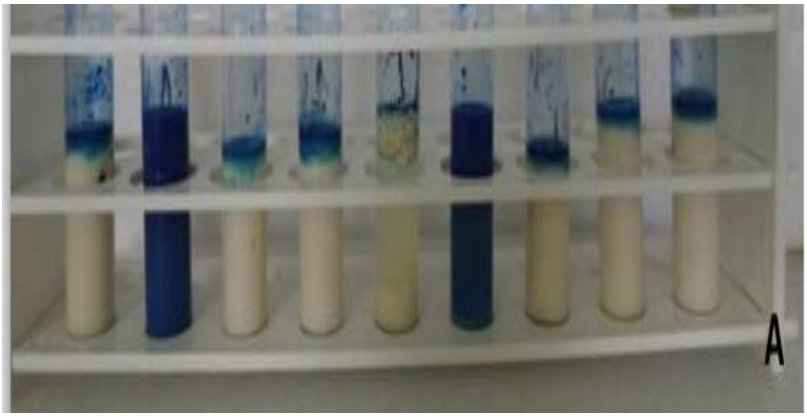

Figure 27. Reductase test or Sherman blue test of *Enterococcus* strains (Houbad, 2015)

3-8- Fermentation profiles (carbohydrate utilization tests) (differential test)

Carbohydrate utilization is a classical method to distinguish LAB at the genus and species level. Different LAB can ferment various sugars, producing acid (and sometimes gas) as end products. By testing several carbohydrates, a "fermentation profile" is established that helps to identify and differentiate *Lactococcus* from *Enterococcus* (Tohno et al., 2012).

a. Principle

Bacteria are grown in MRS medium, free of yeast and meat extract, containing a single carbohydrate source (e.g., glucose, lactose, mannitol, raffinose, arabinose), along with a pH indicator such as bromocresol purple. If the sugar is fermented, acids are produced that lower the pH, causing the indicator to change color.

b. Inoculation

For this assay, the inoculum is prepared from a fresh culture of the test strain rather than directly from a colony. The culture is first centrifuged, and the cells are washed twice with phosphate buffer to remove residual nutrients. The resulting pellet is then resuspended in phosphate-buffered saline (PBS), and the bacterial suspension is adjusted so that it represents 1% of the final test medium.

Each carbohydrate to be studied is added to the basal medium at the same final concentration. The prepared mixtures are inoculated with the standardized bacterial suspension and incubated at 30 °C for 24-48 hours. This procedure ensures uniform conditions and allows reliable comparison of fermentation abilities across different sugars.

c. Results interpretation

Positive fermentation: Medium turns yellow, indicating acid production.
Negative fermentation**:** Medium remains red/orange, indicating no acid formation.

Typical patterns:

Lactococcus → usually ferment lactose and glucose but show a narrower sugar range**.**
Enterococcus → ferment a broader range of carbohydrates, including mannitol, sorbitol, and others.
It is important to note that the sugar fermentation profile depends strongly on the strain. Two isolates belonging to the same species may exhibit different carbohydrate utilization patterns, which reflects the genetic and ecological diversity of LAB.

Table 1. Carbohydrate profile comparison between *Lactococcus* spp. and *Enterococcus* spp. (Manero and Blanch, 1999, Passerini et al., 2013)

Carbohydrate / Sugar	***Lactococcus* spp. (typical)**	***Enterococcus* spp. (typical)**	**Notes / References**
Glucose	Fermented → acid produced	Fermented → acid produced	Both are lactic acid bacteria; Enterococcus uses fermentation
Lactose	Fermented (since many *Lactococcus* are dairy-associated)	Many can ferment lactose, though variability exists	*Lactococcus* is well-known for lactose fermentation in dairy
Mannitol	In many strains: no or weak fermentation	Many *Enterococcus* strains ferment mannitol	The ability of enterococci to use a broader set of sugars (e.g., mannitol) is often noted.
Sucrose	Some *Lactococcus* strains ferment sucrose	Many *Enterococcus* ferment sucrose	*Enterococcus* often show more diverse sugar utilization profile
Raffinose	Some *Lactococcus* (strain-dependent)	Some *Enterococcus* can ferment raffinose	Metabolic diversity is strain-dependent

Galactose	Many *Lactococcus* ferment galactose (especially dairy strains)	Some *Enterococcus* – variable	The galactose pathway is often present in dairy *Lactococcus*
Xylose / Arabinose / Pentoses	Rarely fermented or weak	Some *Enterococcus* can ferment pentoses in certain strains	*Enterococcus* shows somewhat broader carbohydrate metabolism in many studies.

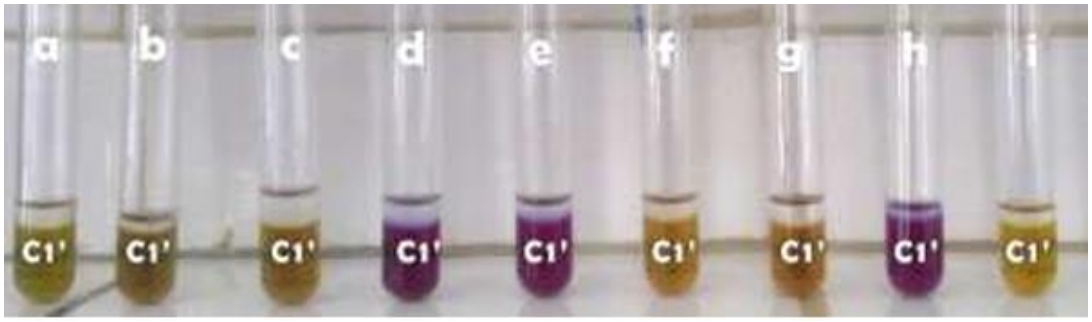

Figure 28. Example of fermentative profile of LAB strain using a classical gallery.

a: glucose, b: lactose, c: sucrose, d: rhamnose, e: esculin, f: galactose, g: maltose, h: mannitol, i: xylose. Yellow: positive reaction; purpule/orange: negative reaction (Zarour, 2010)