

Practical Microbiology

**Cihan University
Medical Laboratory Analysis**

Lab 7: Differential staining (Gram staining)

Lec: Karokh Ali Khdir

MSc: Microbiology

Second stage (1st semester)

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Objective

- ❖ **Students should be able to understand:**
- ✓ Principle of Gram staining.
- ✓ Gram positive and Gram negative bacteria (due to cell wall composition).
- ✓ Steps in Gram staining.
- ✓ Variation, limitation & precaution in Gram staining technique.

Gram staining

- It was developed by Danish physician Hans Christian Gram in 1884.
- It is still the cornerstone of bacterial identification.
- Differentiate bacteria into **Gram positive & Gram negative** based on chemical composition of their cell wall so called differential.
- Used two stains instead of one stain. The first stain (**primary stain**) and second stain (**counter stain**).

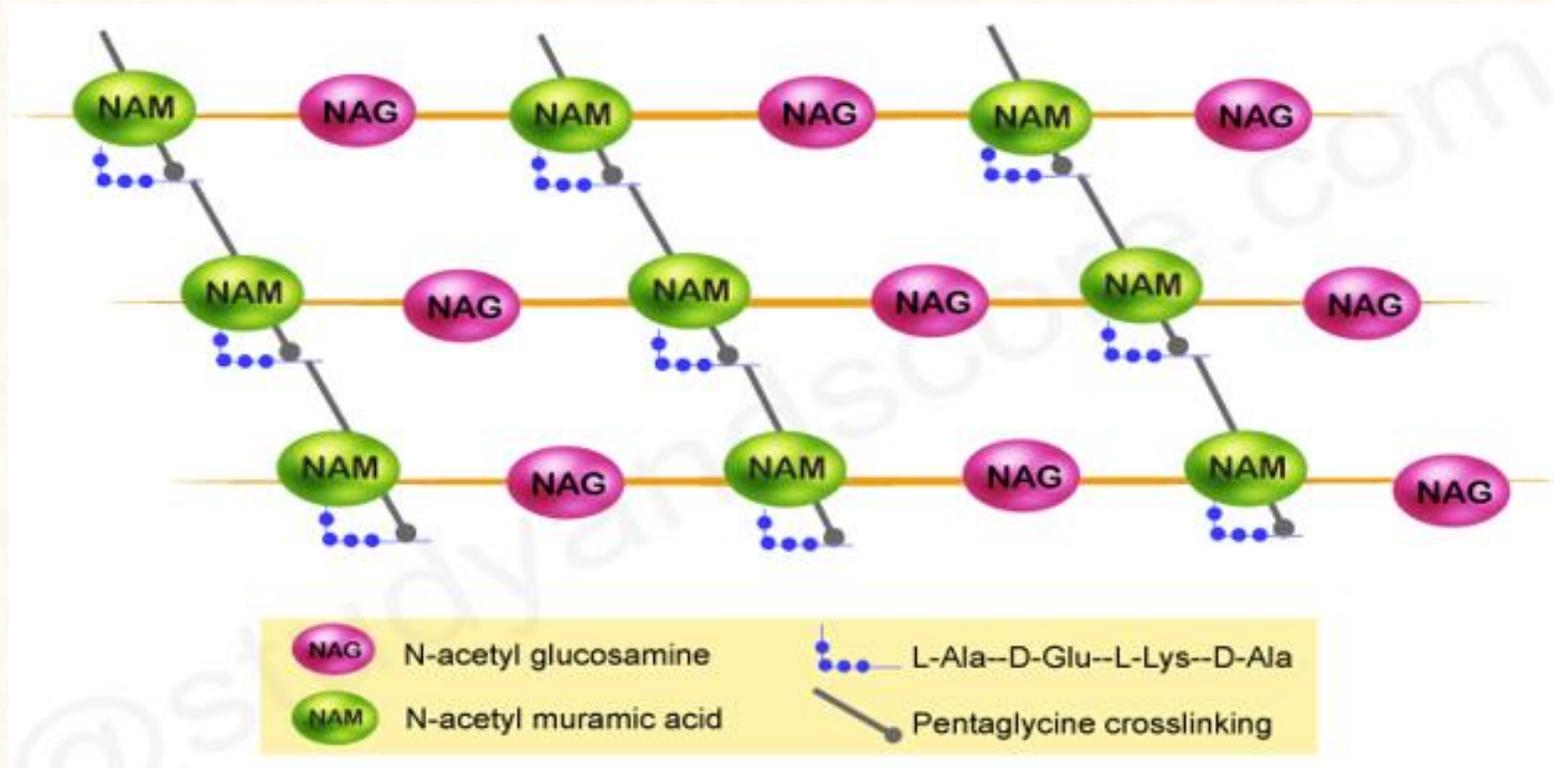


Hans
Christian
Gram
1853-1938

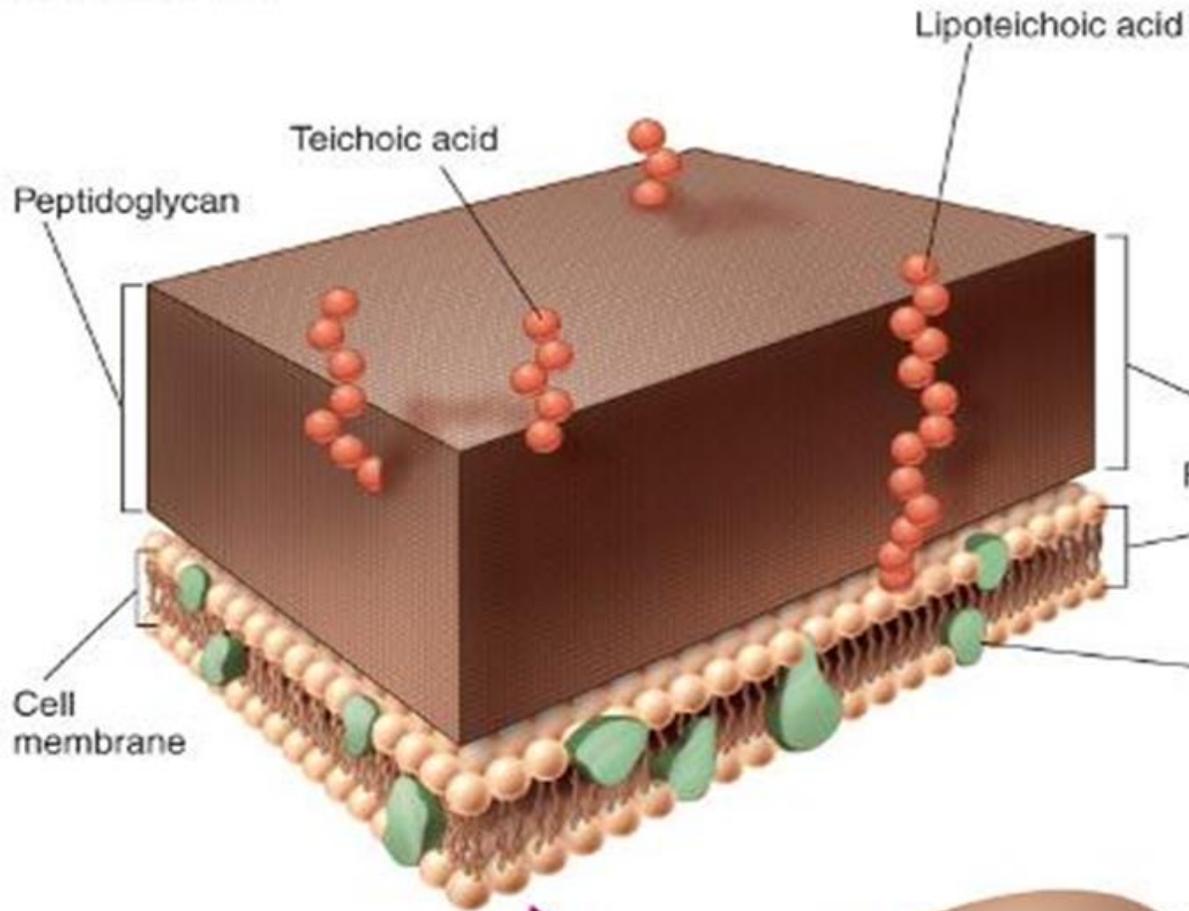


Cell wall in Gram+ & Gram-

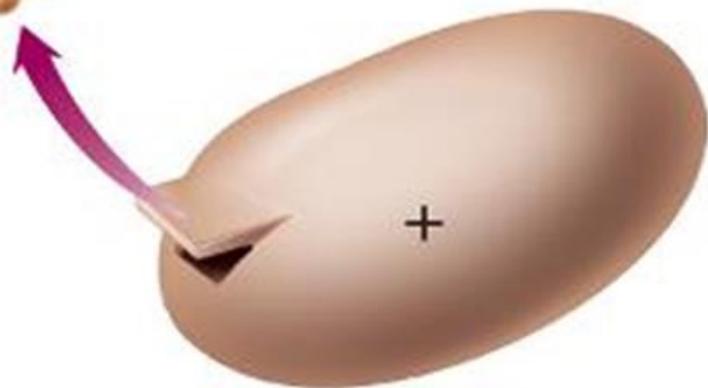
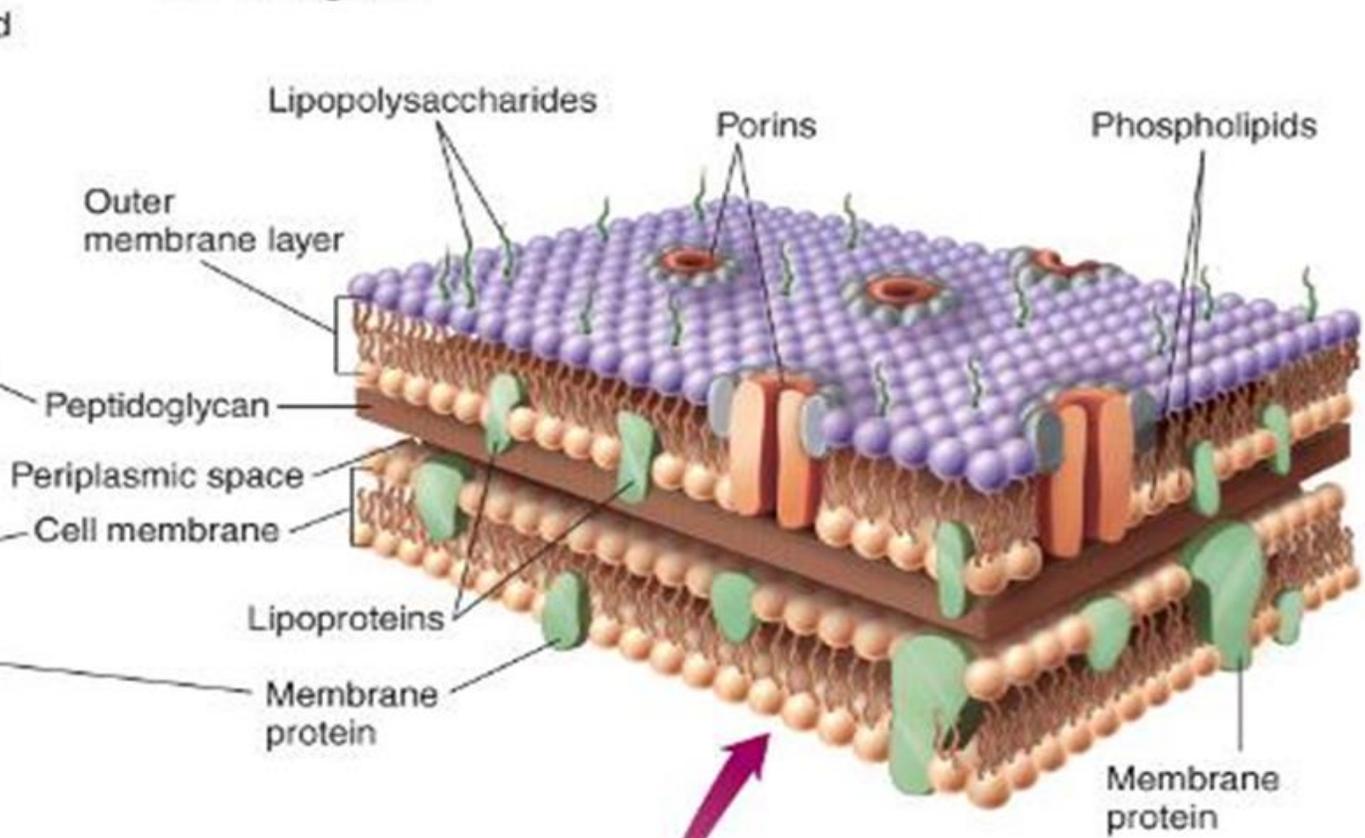
- Cell wall composed of Peptidoglycan that has both **protein** and **polysaccharide**
- The polysaccharide portion is made of two alternating sugars, ***N*-acetylglucosamine (NAG)** and ***N*-acetylmuramic acid (NAM)**
- The protein (peptide) portion consists of a short chain of amino acids that link layers of peptidoglycan via NAM, this link called peptide bridge or peptide cross bridge.



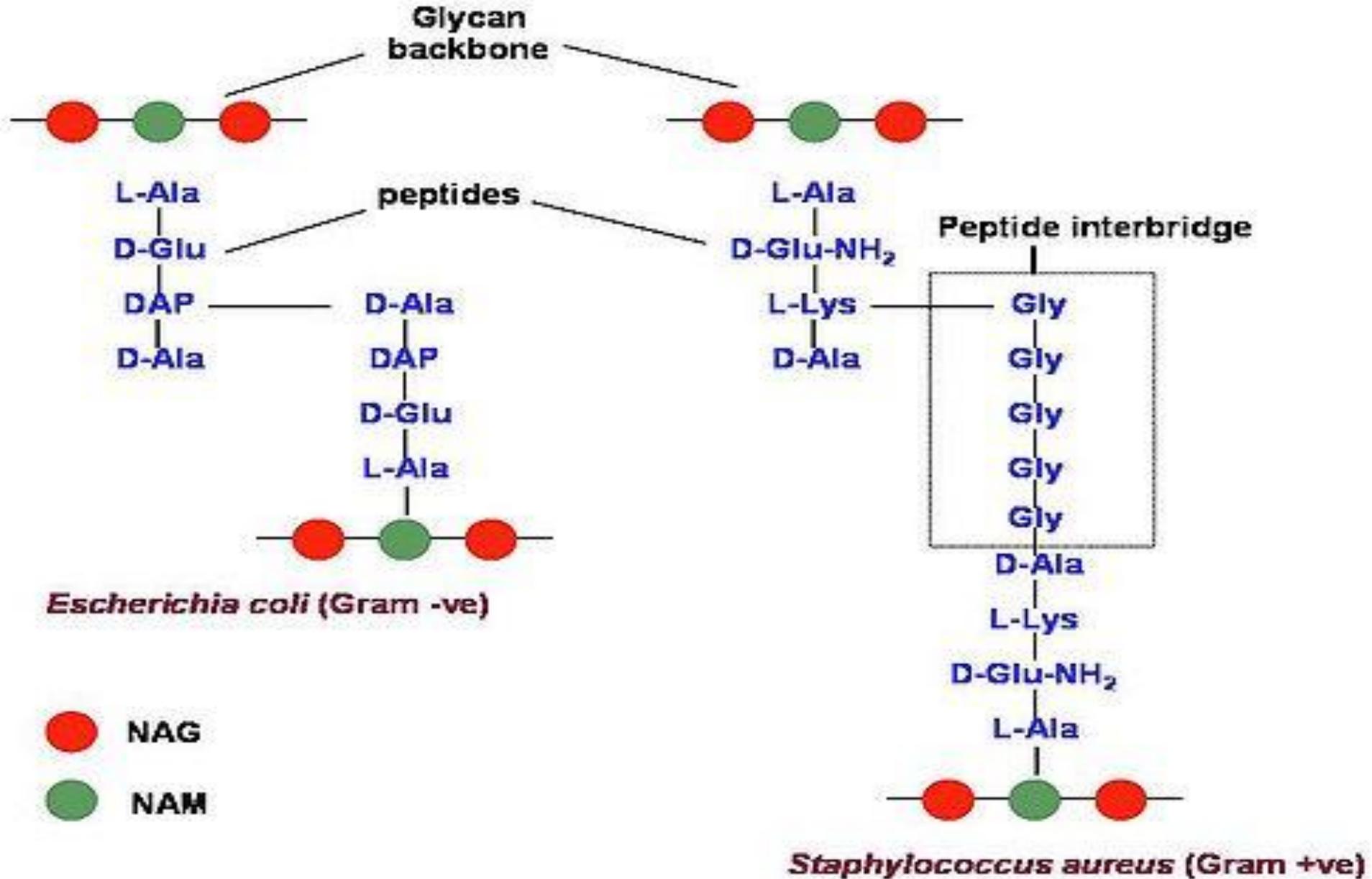
Gram-Positive



Gram-Negative



Interpeptide bridge



Gram staining

- Using two dyes in sequence, each of a different color.
- bacteria fall into two groups.
- The first group retains the color of the primary dye (**crystal violet**) these are called **Gram positive (purple or blue color)**.
- The second group loses the first dye when washed in a decolorizing alcoholic solution but then takes on the color of the second dye such as **safranin** these are called **Gram negative (pink)**.

➤ An **iodine** solution is used as a mordant (a chemical that fixes a dye in or on a substance by combining with the dye to form an insoluble compound (CV-I complex) for the first stain.

Procedure

- ✓ Smear Preparation, fix sample on a slide with heat, allow it to cool before applying the stain.
- ✓ Flood air-dried, smear with **crystal violet** staining reagent for 1 minute.
- ✓ Wash slide in a gentle and indirect stream of tap water.
- ✓ Flood slide with the mordant: **Gram's iodine**, wait for 1 minute.
- ✓ Wash slide in a gentle and indirect stream of tap water.
- ✓ Flood slide with **decolorizing agent (95% ethanol)**. Wait for 15 seconds.
- ✓ Wash with tap water.
- ✓ Flood slide with a counterstain, **safranin**, wait for 30 seconds to 1 minute.
- ✓ Wash slide in a gentle and indirect stream of tap water.
- ✓ Blot dry with absorbent paper or air dry.
- ✓ Observe the slide under 4 x & 100x oil immersion lenses.

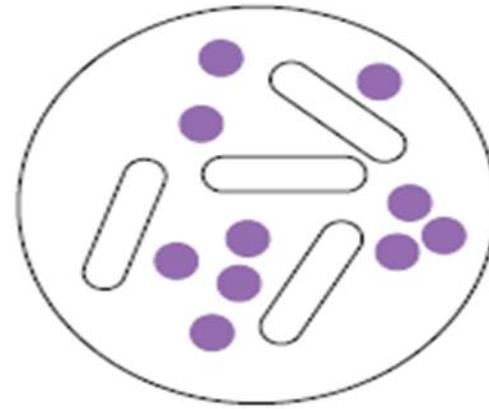
Gram staining is used for detecting whether the bacteria is **G+ve** or **G-ve**, in addition to showing **size, shape, and arrangement**.



(a) Application of crystal violet:
All cells are purple.



(b) Application of Gram's iodine:
All cells are purple-black.

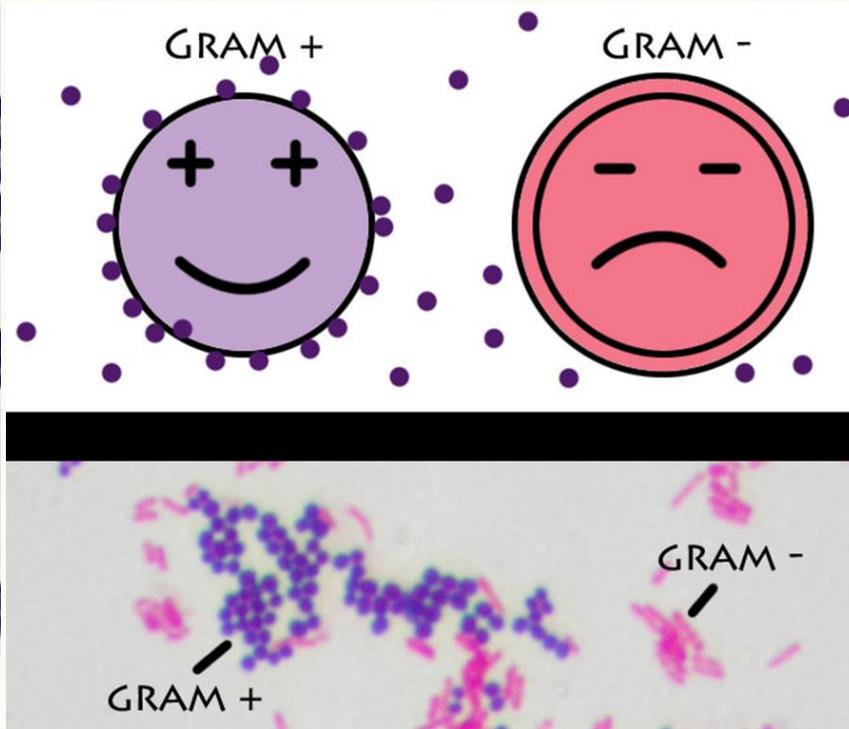
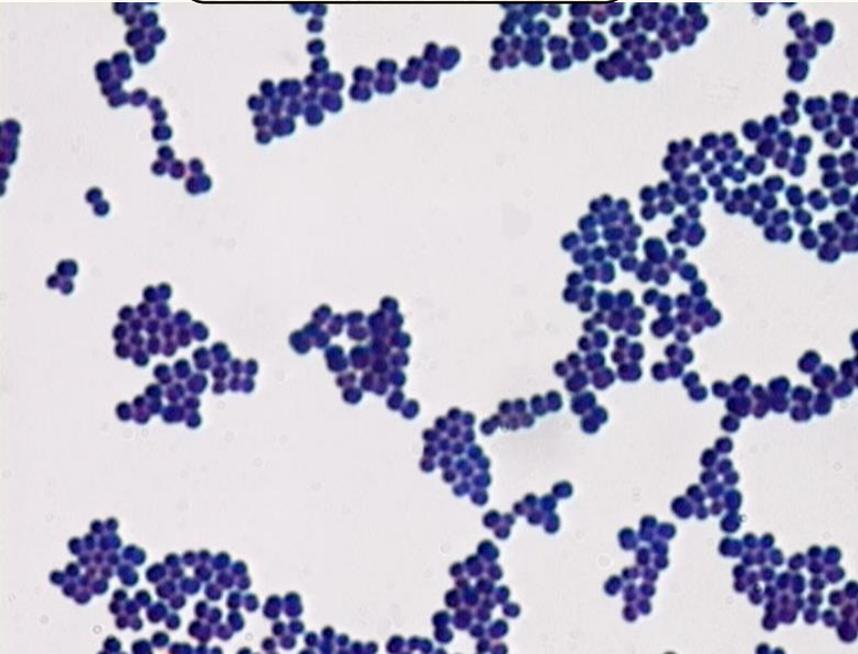


(c) 95% alcohol wash:
Gram-positive cells are purple; gram-negative cells are colorless.

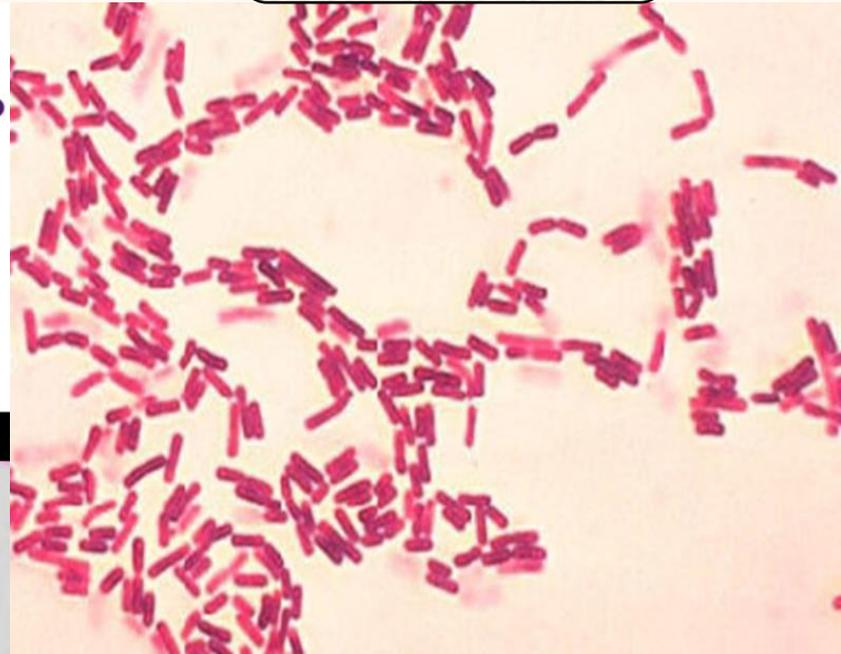


(d) Application of safranin:
Gram-positive cells are purple; gram-negative cells are pink.

Gram +



Gram -



Gram staining

- ✓ Nowadays, Gram staining is still in use:
- ✓ Identification of bacteria from environmental samples.
- ✓ Bacterial classification.
- ✓ Diagnosis of clinically important bacteria.



List of Gram+ & Gram- bacteria

➤ Gram positive

Cocci: *Staphylococcus, Streptococcus, Enterococcus, Micrococcus.*

Bacilli: *Bacillus, Clostridium, Corynebacterium, Listeria, Actinomyces.*

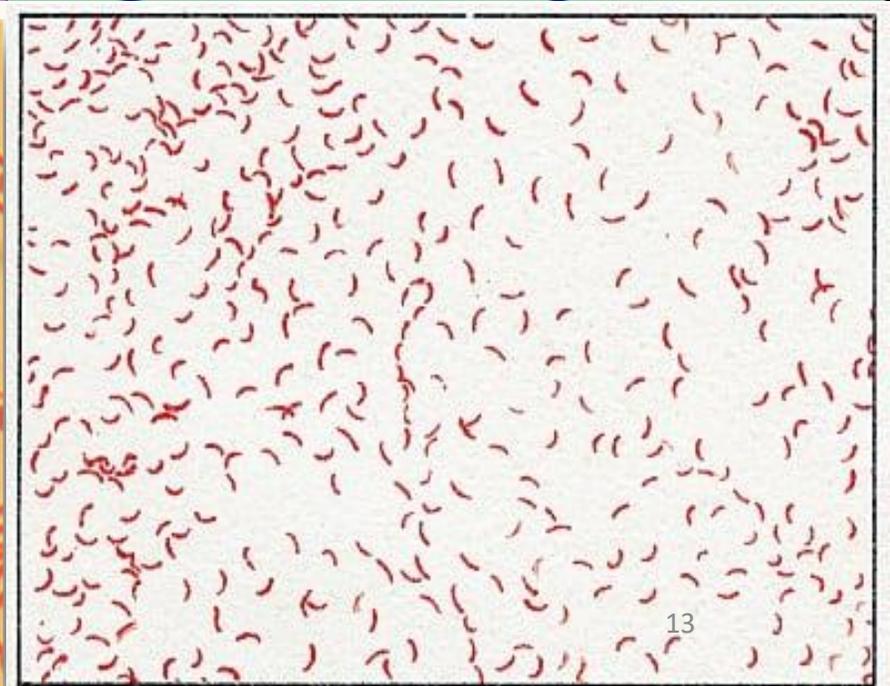
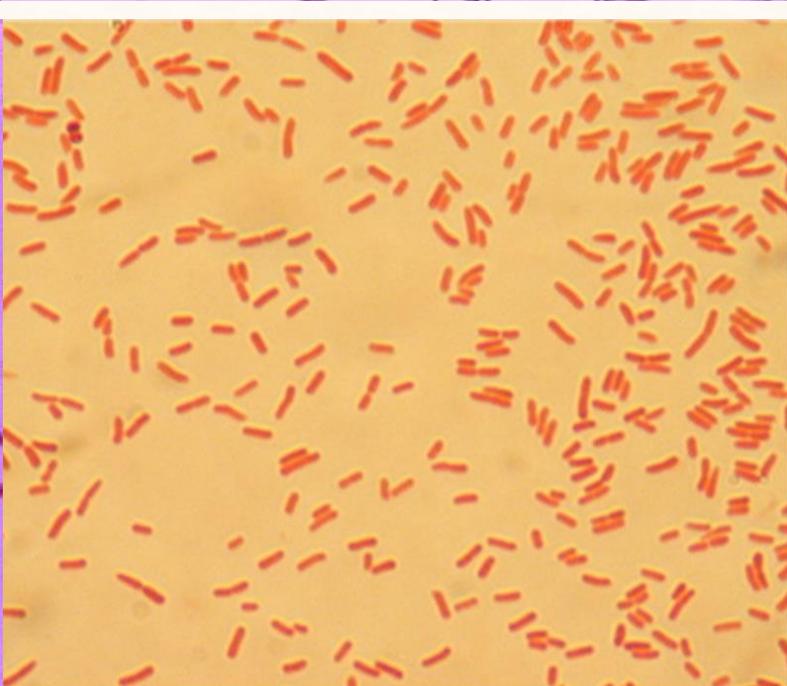
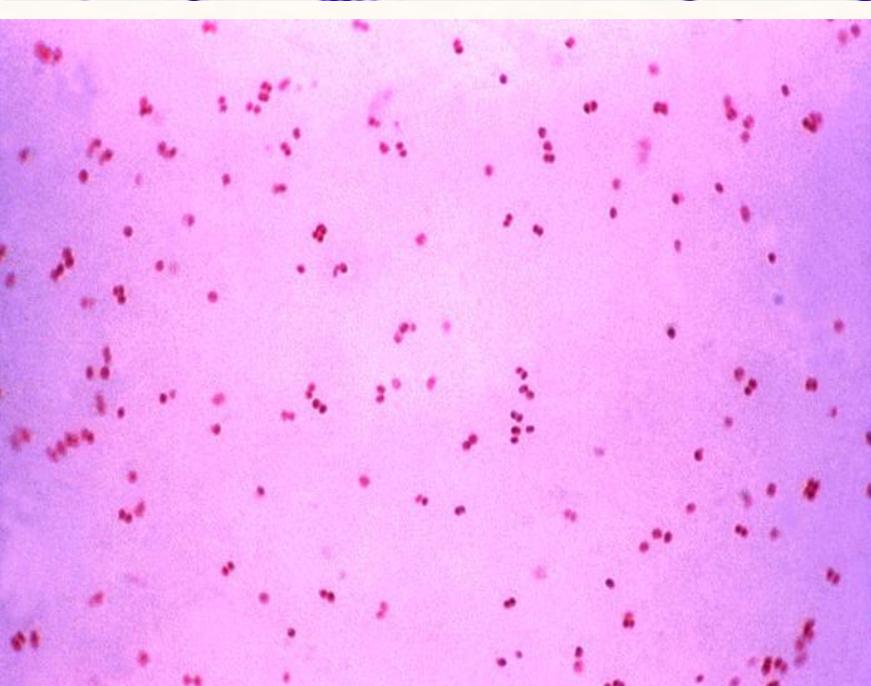
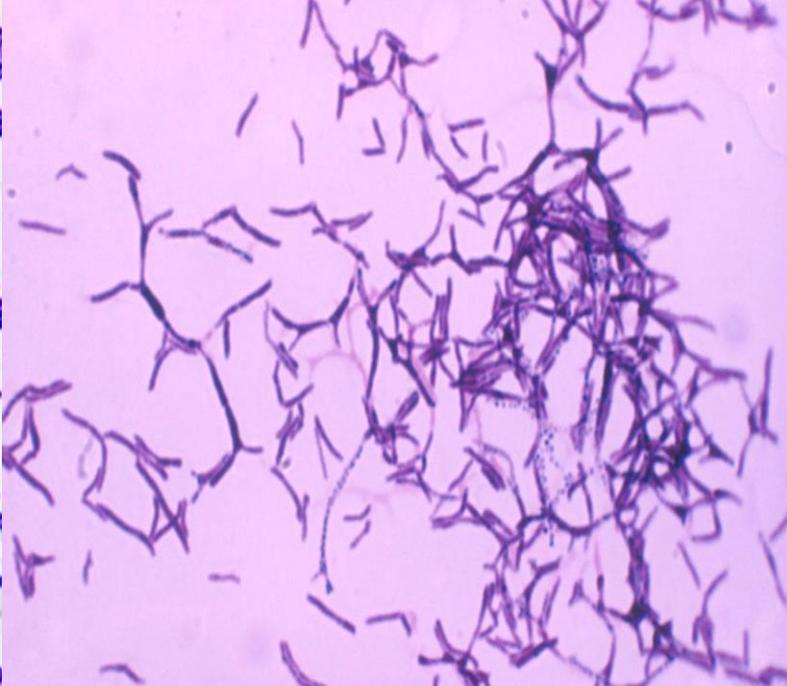
➤ Gram negative

Cocci: *Neisseria.*

Bacilli: Enterobacteriaceae (*Escherichia coli, Klebsiella*), *Pseudomonas, Acinetobacter.*

Curved rod: *Vibrio cholerae.*

Helical: *Helicobacter pylori.*



Limitation & precaution

- ✓ To be visible on a slide, organisms that stain by the Gram staining must be present in concentrations of about 10^4 to 10^5 cells per ml.
- ✓ Not all bacteria can be seen in the Gram stain (*Mycobacterium*, *Mycoplasma*, *Rickettsia*, *Chlamydiae*).
- ✓ Always check new batches of stain and reagents.
- ✓ Fresh culture of 18-24 hours must be used for Gram staining, otherwise the results will be variable or false.
- ✓ Gram staining must be done for well isolated colony or pure culture, especially in the case of identification process.

Variation in Gram staining

❖ **Gram-positive bacteria may be appear as Gram negative:**

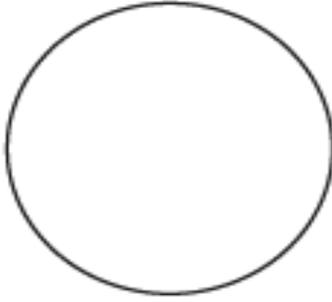
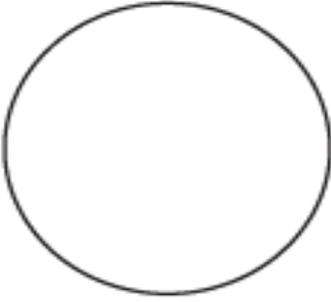
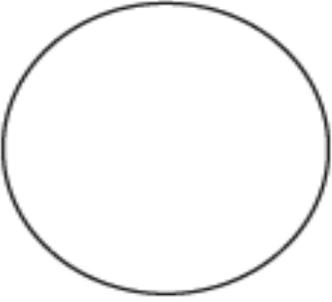
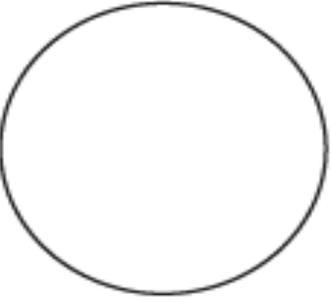
- ✓ Cell wall damage (antibiotic therapy or excessive heat fixation).
- ✓ Over decolorization of the smear.
- ✓ Use of an Iodine solution which is too old.
- ✓ Smear has been prepared from an old cultures.

❖ **Gram negative may be appear as gram positive:**

- ✓ When smear is too thick, Gram-negative bacteria may not be fully decolorized.
- ✓ Under decolorization of smear.

Lab activity

- Gram staining must be done for several colonies that isolated previously.
- Observe whether the bacteria are Gram positive or negative.
- Morphology of the bacteria, whether cocci, rods, curved.
- Cell arrangements, whether cluster, chain, pleomorphic.

	<i>E. coli</i>	<i>B. cereus</i>	<i>S. aureus</i>	<i>Mixture</i>
Draw a representative field.				
Cell morphology:				
Shape	_____	_____	_____	_____
Arrangement	_____	_____	_____	_____
Cell color	_____	_____	_____	_____
Gram reaction	_____	_____	_____	_____

References

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