



FOOD SAFETY LAB / MILK QUALITY
IMPROVEMENT PROGRAM



Standard Operating Procedure

Title: Gram Stain

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Gram Stain

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SECTION 1 INTRODUCTION

1.1 Purpose

The purpose of this experiment is to perform the Gram stain procedure on one or more pure cultures of bacteria. The Gram stain is a classic microbiological test that quickly provides information regarding the morphology and cell wall construction of bacterial cells.

1.2 Scope

This SOP applies to the Milk Quality Improvement Program Laboratory and to the Food Safety Lab.

1.3 Definitions

- **Gram Stain:** a method of staining used to distinguish and classify bacterial species into two large groups: Gram-positive bacteria and Gram-negative bacteria, based on their differential retention of dyes.
- **Gram positive:** Gram-positive bacteria generally have a single membrane (monoderm) surrounded by a thick peptidoglycan layer. These cells retain the crystal violet dye and appear purple. Gram-positive bacteria are usually from the phylum Firmicutes.
- **Gram negative:** Gram-negative bacteria generally possess a thin layer of peptidoglycan between two membranes (diderms). Most bacterial phyla are Gram-negative. Gram-negative bacteria do not retain the crystal violet dye and appear pink due to the safranin counterstain.
- **Gram variable:** Some bacteria do not stain consistently Gram positive or Gram negative, or their Gram status depends on the culture age and other conditions. *Paenibacillus* often stain Gram negative, despite being Firmicutes, which typically (though not always) stain Gram positive. See the Troubleshooting section for additional information.

1.4 Safety

Crystal violet, Gram's iodine, and safranin all stain clothing and skin. Wear appropriate PPE to avoid exposure. Read the SDS for the Gram stain components to learn more about safety precautions, but the Gram stain procedure is safe enough to perform on the benchtop.



Use caution when passing the slide through the flame of a Bunsen burner to heat-fix the smear. Use a clothespin or a pair of forceps to hold the slide and put it down on the bench to cool for several seconds before touching it.

If using methanol to fix the smear, be aware of its hazards, which you can learn about in its SDS and in right-to-know labels on any non-original containers. Always perform this fixation in the chemical fume hood.

SECTION 2 MATERIALS

- **Bacterial culture**
 - Culture should be less than 48 hours old, and preferably less than 24. Old cells produce unreliable Gram stain results.
 - For bacteria in pure culture on solid media, proceed with instructions below.
 - For bacteria in pure culture in liquid media, centrifuge the culture at $10,000\times g$ for 1 min and resuspend the pellet in an equal or slightly smaller volume of dH₂O. Use this solution to prepare the smear.
- **Gram stain kit, containing:**
 - Crystal violet solution
 - Gram's iodine solution
 - Decolorizer solution (alternately, 70% ethanol may be used)
 - Safranin solution
- Glass microscope slides
- Sterile dH₂O
- Methanol (optional)
- P10 micropipette and pipette tips
- Toothpicks
- Stain tray and clothespins
- Grease pencil
- Wash bottle filled with dH₂O
- Bibulous paper

- Compound light microscope with 100 \times oil immersion objective lens
- Mobile phone with camera to photograph results (optional)



SECTION 3 PROCEDURES

1. Prepare the smear.
 - a. Draw up to 3 evenly spaced circles approximately 1.5 cm in diameter on a clean glass microscope slide using a grease pencil. Marker will not work, as the Gram-stain solvents will dissolve and remove the pigment.
 - b. Pipette 5-10 μL of dH_2O into the circle.
 - c. Using a toothpick, gently touch an isolated bacterial colony on a petri dish, then vigorously mix the piece of colony into the drop of water on the slide using the toothpick. Ensure the water covers the entire area inside the circle.
 - i. It is important to avoid picking too much of the colony. Crowded cells will not stain properly and will make interpretation of results impossible.
 - d. Allow the smear to dry uncovered at room temperature.
 - e. Fix the cells on the smear using one of the following techniques:
 - i. Slowly pass the bottom of the slide over the tip of the cone of the flame of a lit Bunsen burner three times. Allow the slide to cool before proceeding.
 - ii. Working in the chemical fume hood, place one drop of 100% methanol directly on top of the smear and allow to evaporate fully before proceeding.
 - iii. Either of these steps denatures cell surface proteins, affixing them to the glass slide and preventing the cells from being washed away during the staining procedure.
 - f. Place the slide in a clothespin on top of a staining tray.
 - g. Drop crystal violet onto each smear to cover entirely. Wait 1 min.
 - h. Use the dH_2O wash bottle to gently rinse the crystal violet solution off of the smear and the front and back of the slide. Rinsing too forcefully could remove the affixed cells. Tap the slide gently on the staining tray to knock of excess water.
 - i. Drop Gram's iodone onto each smear to cover entirely. Wait 1 min.
 - j. Use the dH_2O wash bottle to gently rinse the iodine solution off of the smear and the front and back of the slide. Tap the slide gently on the staining tray to knock of excess water.
 - k. While holding the slide up vertically in front of a light-colored background, drip decolorizer over the smear only just until you see the crystal violet dye stop dissolving off of the smear. This step should take no longer than 10-15 seconds. Do not over-decolorize. Tap the slide gently on the staining tray to knock of excess water.
 - l. Immediately use the dH_2O wash bottle to gently rinse the decolorizer solution off of the smear and the front and back of the slide.
 - m. Drop safranin onto each smear to cover entirely. Wait 1 min.
 - n. Use the dH_2O wash bottle to gently rinse the safranin solution off of the smear and the front and back of the slide. Tap the slide gently on the staining tray to knock of excess water.
 - o. Dry the slide by gently blotting between sheets of bibulous paper. Do not rub the smear with the paper.



- p. View the smear using the 100× oil immersion objective on the microscope. Use the grease pencil line surrounding the smear to focus the microscope in the correct plane.
- q. Note the morphology and color of the bacterial cells. Cells that stain purple are Gram positive. Cells that stain pink are Gram negative.

High-Throughput:

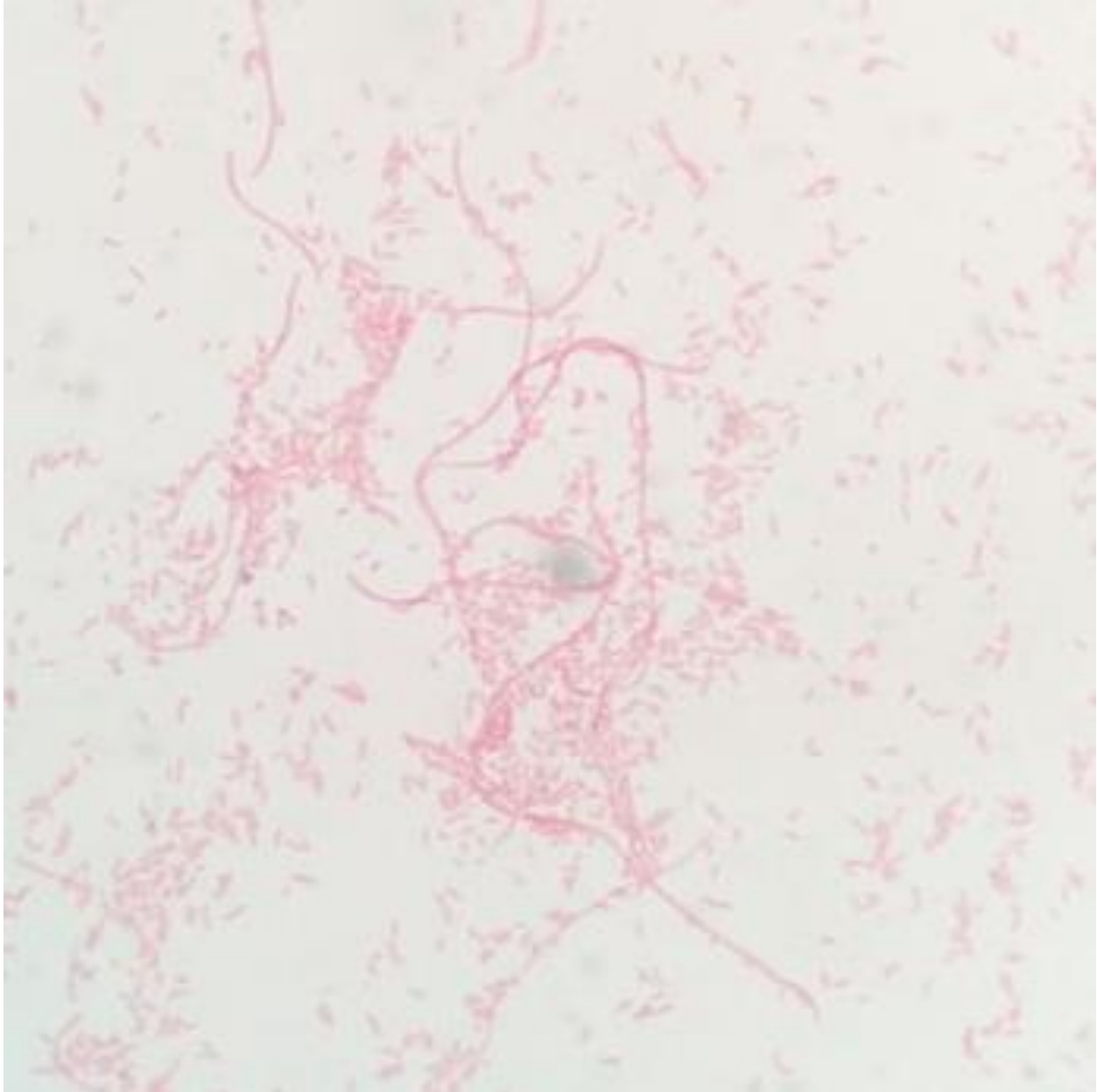
- Large numbers of Gram stains can be performed at once by placing slides containing prepared smears in glass slide racks and dipping the racks successively into reservoirs containing stain reagents in the sequence described above. Slide racks and stain reservoirs are stored with the Gram stain reagents.

Waste Disposal:

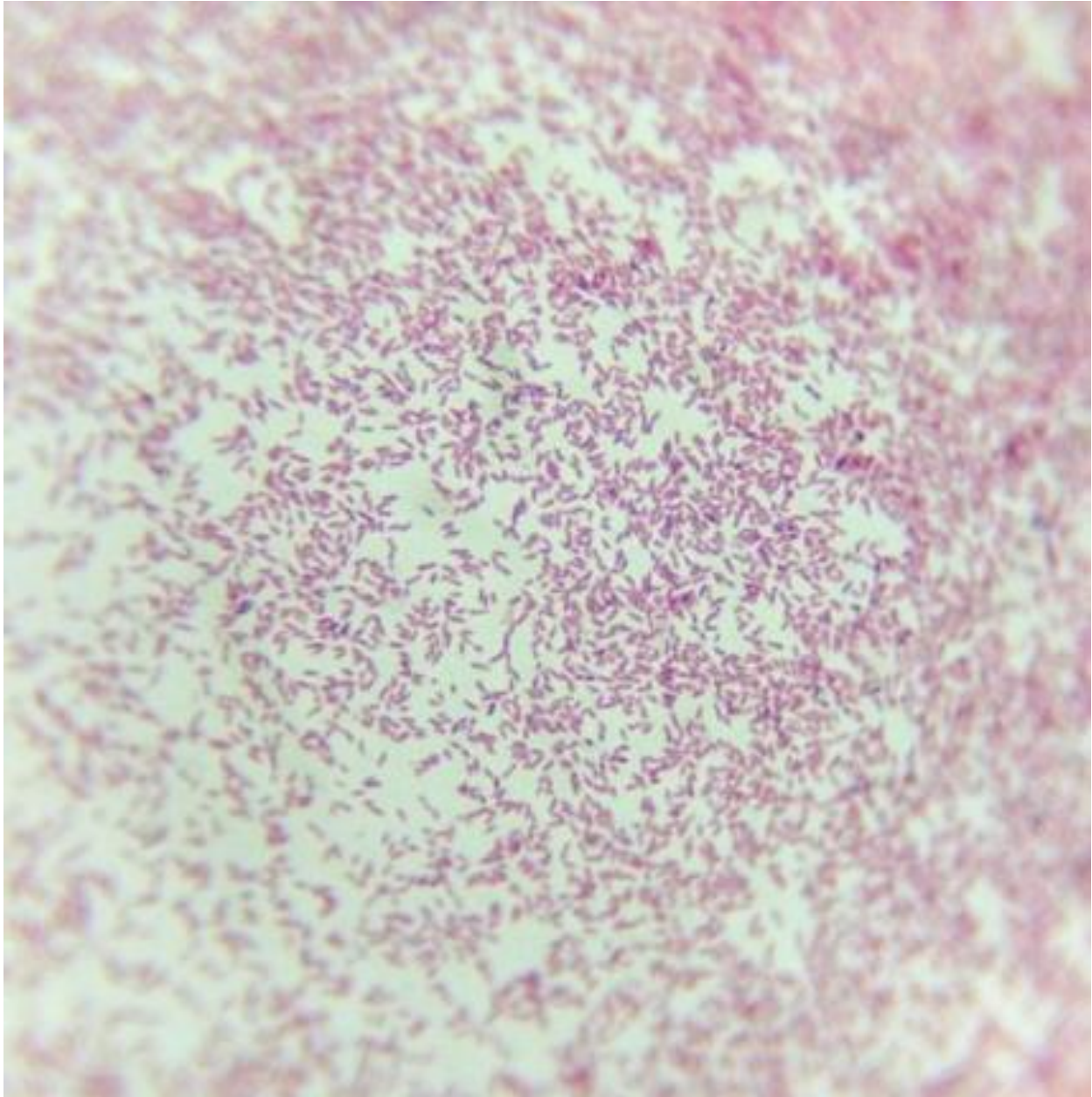
- Small volumes of Gram stain waste can be safely rinsed down the drain with copious quantities of water. Be careful not to stain the interior of the sink with this waste. Bleach (applied while wearing proper PPE) can be used to clean the sink after staining with Gram stain waste. 70% ethanol will remove Gram stain reagent stains from benchtops.
- Large volumes of Gram stain waste (from performing more than a few stains at once) should be collected and disposed of as chemical waste with Cornell EH&S. See Sherry or Alan for instructions on how to do this.
- Gram stain slides can either be cleaned and reused or disposed of as contaminated sharps waste (red plastic containers).



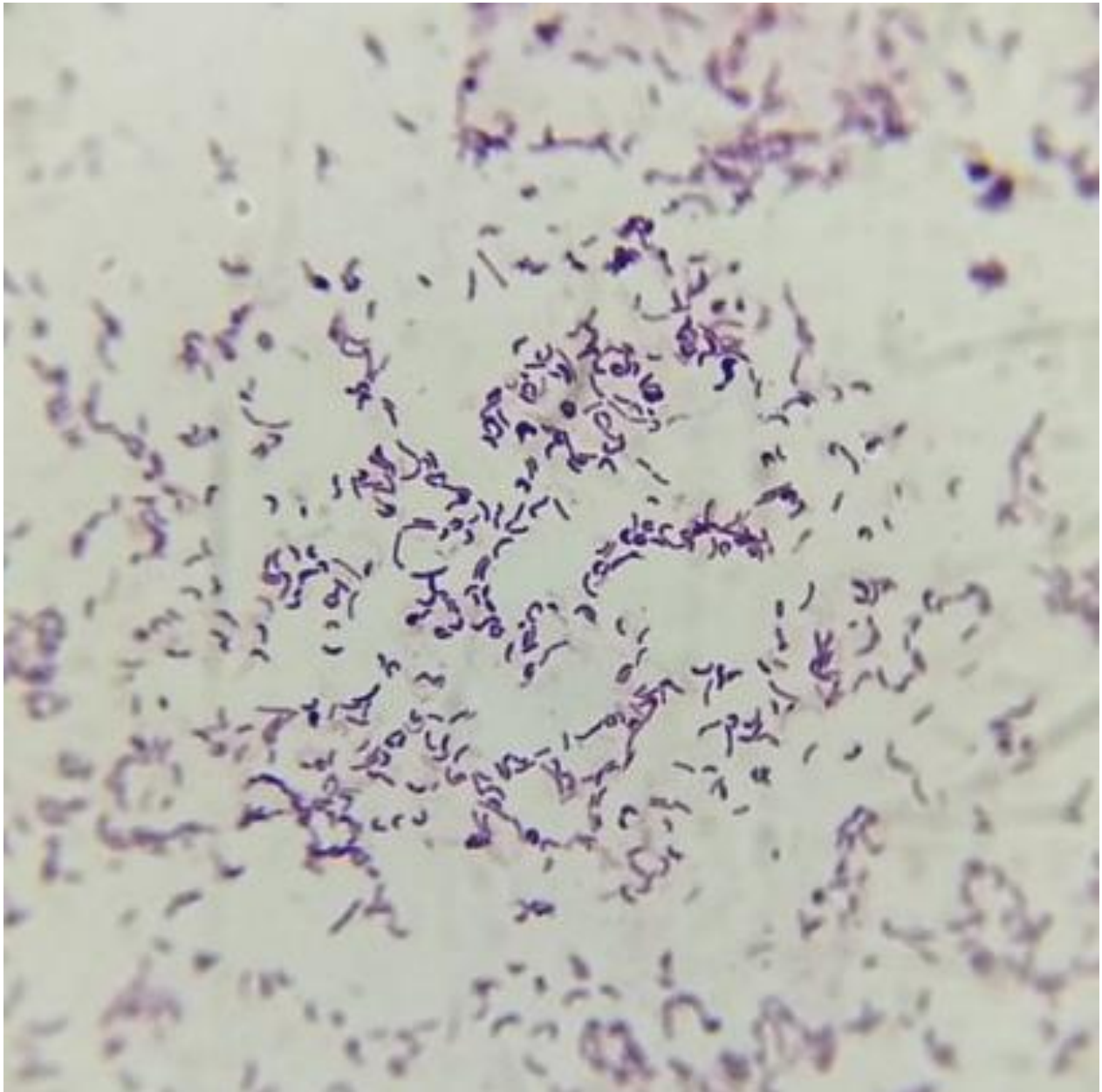
Examples:



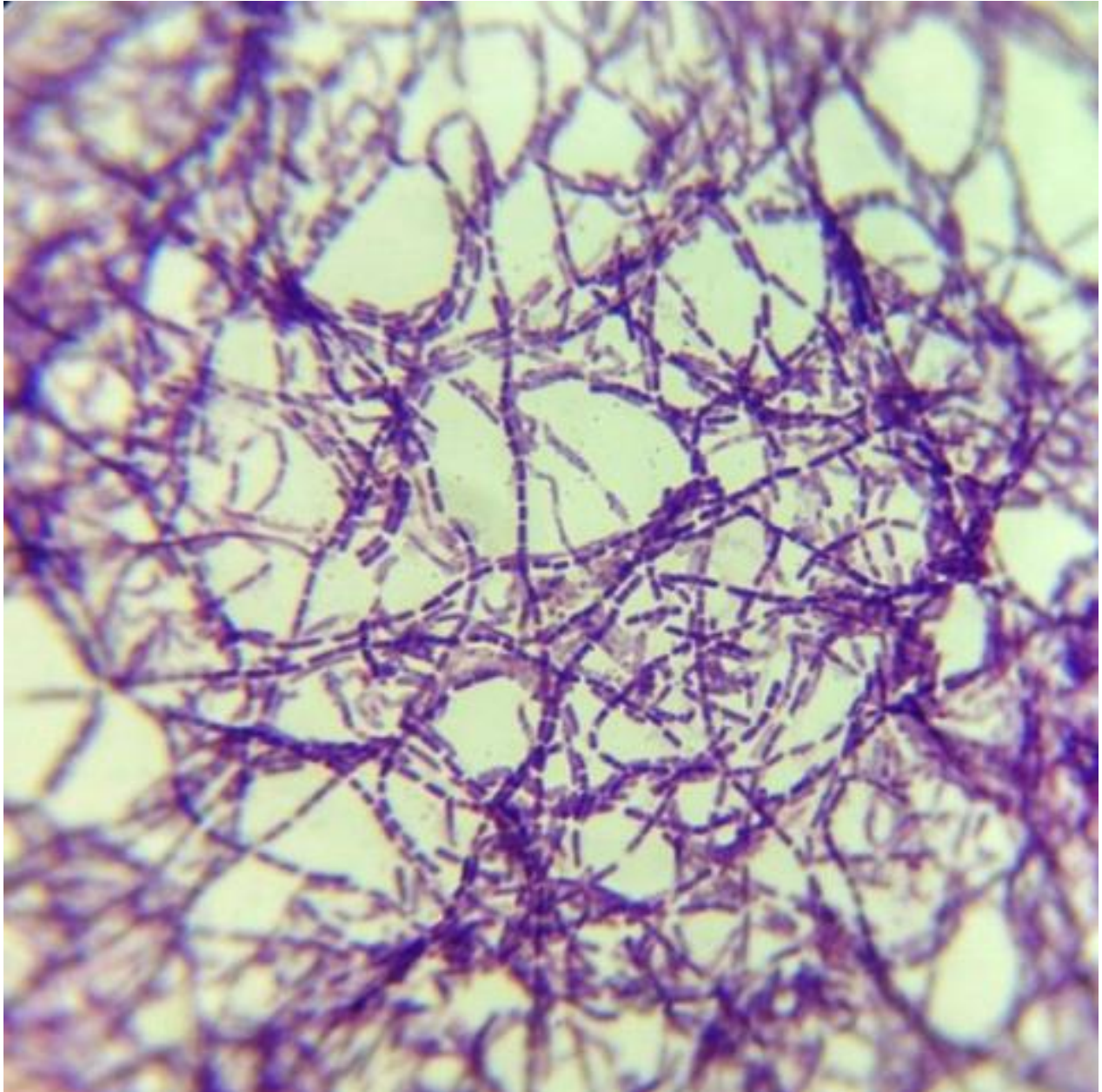
Pseudomonas sp. from pasteurized milk. Gram negative rods singly and in chains.



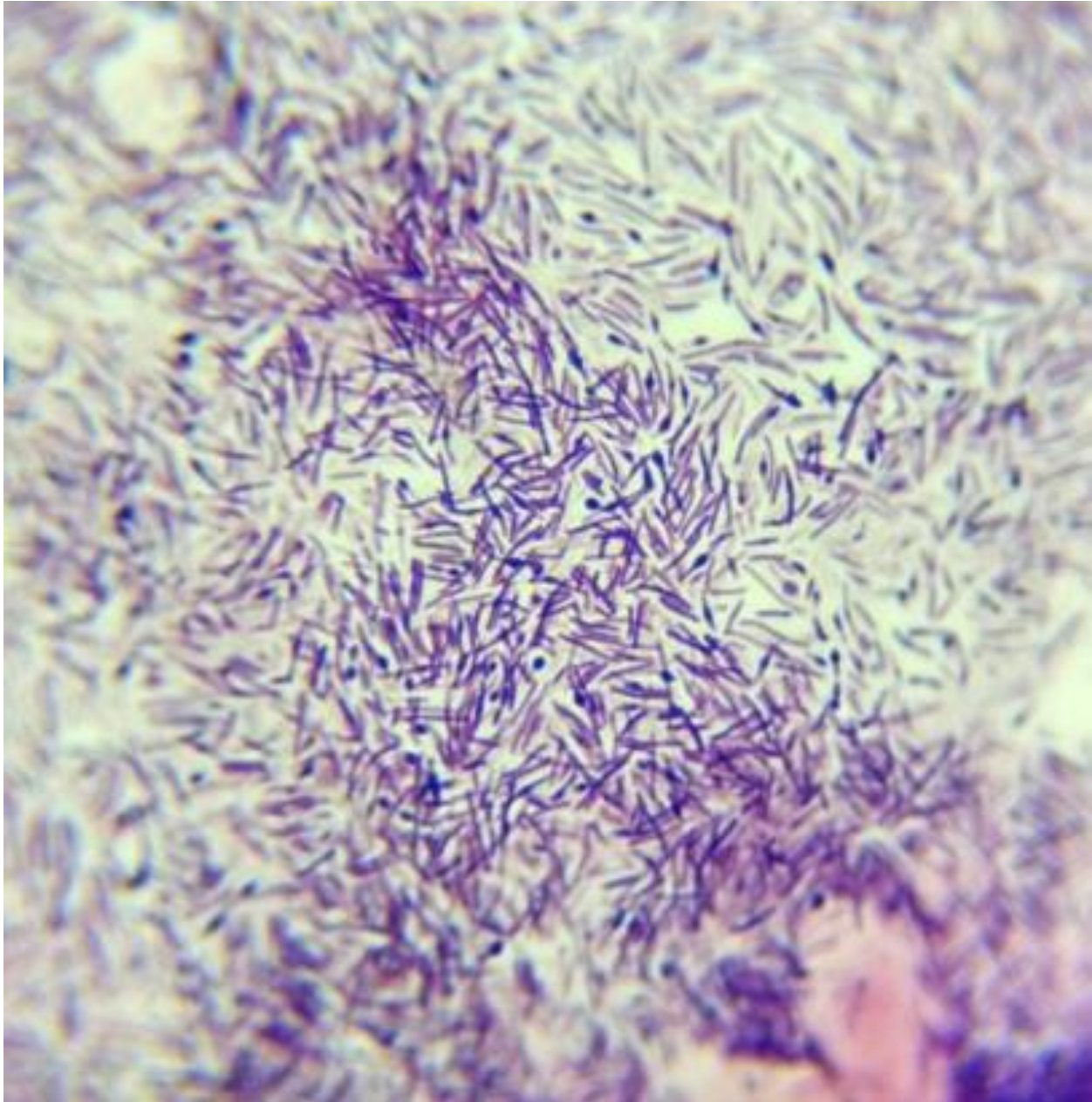
Gram negative rods



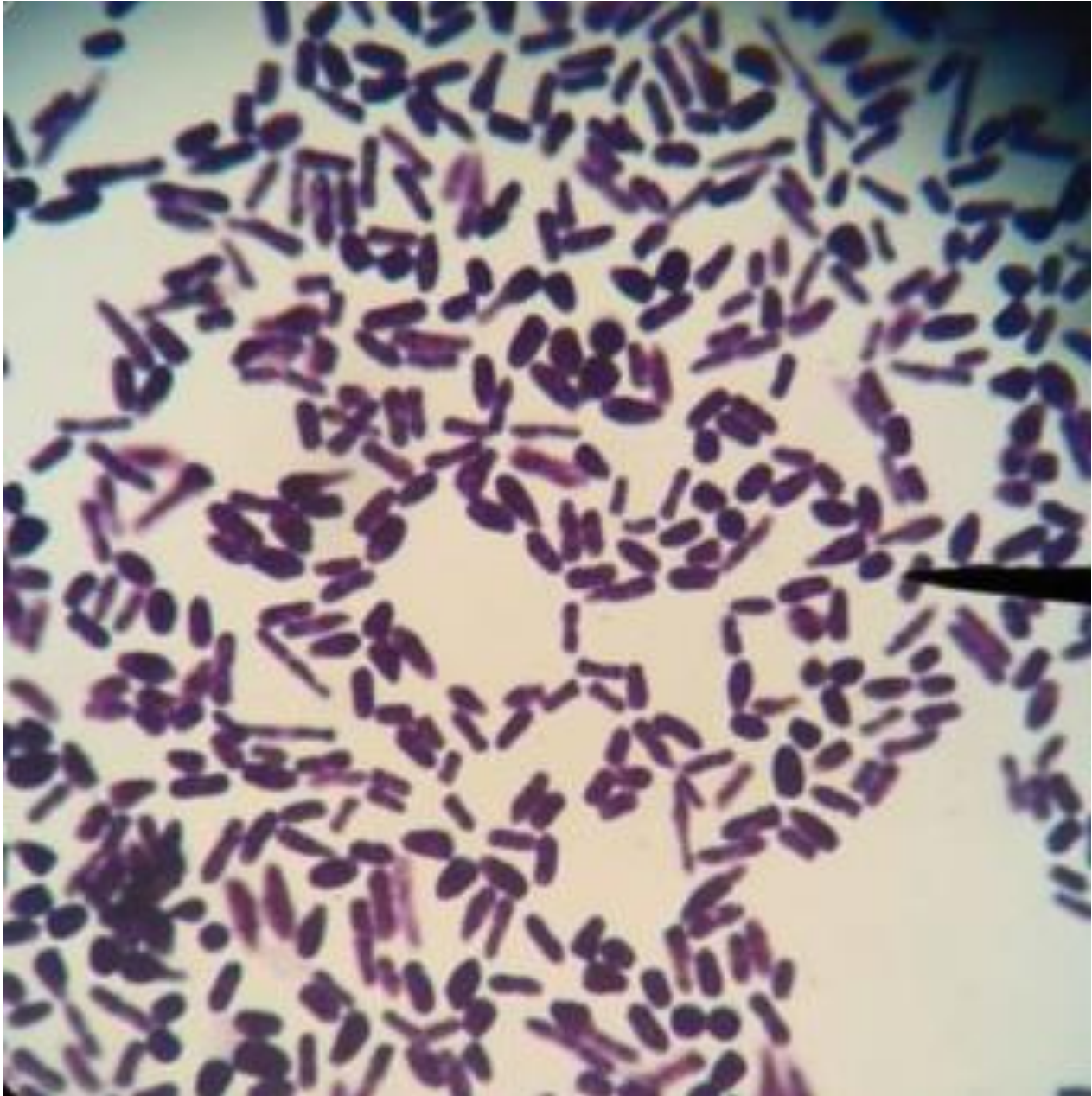
Gram positive rods, probably *Paenibacillus*.



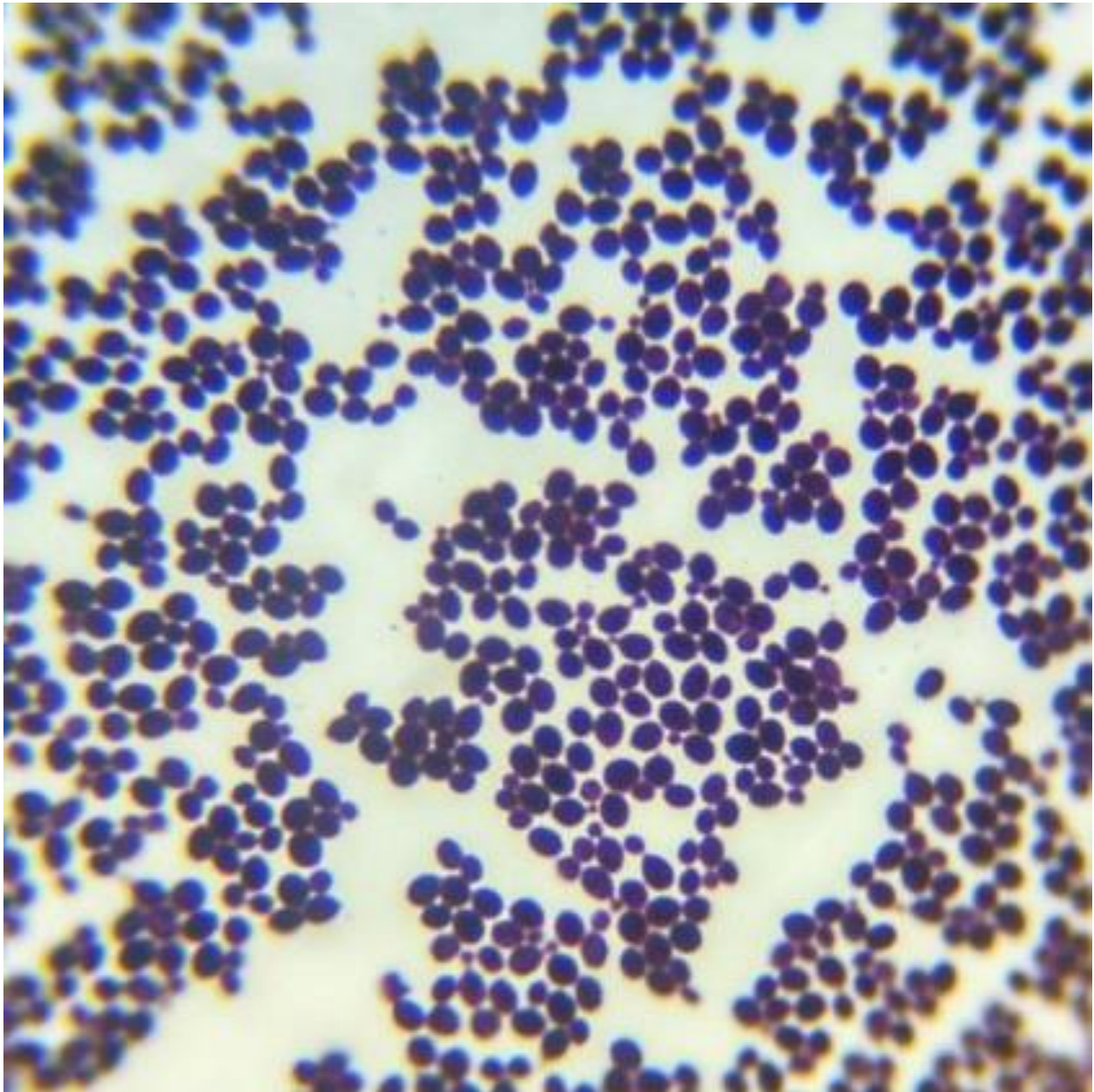
Super cool Gram positive rods in long chains



Gram positive cells with terminal distended endospores (called drumsticks). Spores are a clue, but not a guarantee, that the cells are truly Gram positive.



Yeast. Yeast stain strongly Gram positive and are weird shapes that don't look like bacteria.



Classically shaped, “Mickey Mouse” yeast cells



Me dressed as a Gram stain at my friend Sarah's (BS '14) Halloween-themed wedding



SECTION 4 TROUBLESHOOTING

Smear density: If you make your smear too heavy, you may have difficulty interpreting your stain. Look for the part of the smear with the fewest cells in it while on low magnification, and zoom in on that part to interpret the stain at 1,000× oil immersion magnification. If the entire smear is so densely filled that no individual cells are distinguishable, you'll have to start over with a less heavy smear.

Conversely, if you make your smear too light, you may have difficulty finding any stained cells under the microscope. Again, use low magnification to look for regions of the slide with cells, and focus on the very outer edges of the smear, where it first began to dry – the cell density tends to be heavier here.

If you can find no cells on your completed stain, there could be a few explanations:

1. Your smear was too light
2. The cells were not effectively fixed to the slide. If using heat to fix cells, try heating the slide for slightly longer or try using the methanol method. If you used the methanol method, try using the heat method.
3. You dried the finished stain too vigorously, and the bibulous paper wiped the whole stained smear off of the slide. Start over and be gentler this time.

Gram variability: It is essential to remember that the Gram stain is a phenotypic test. It is a test of the thickness of the bacterial cell wall and its ability to retain the crystal violet stain. That's it. Firmicutes, though they normally stain Gram-positive and are often described as such, are not universally Gram positive. *Paenibacillus* cultures, even when correctly prepared and stained, often stain Gram negative, despite being sporeforming Firmicutes. There is a whole class of bacteria within the Firmicutes called the Negativicutes (yes, really), so named because they (correctly) stain Gram negative. Cultures older than 24 hours will often stain Gram negative due to age affecting the permeability of their cell walls. Don't put too much stock into the results of a Gram stain unless you have other factors suggesting that the results are correct, such as:

- The bacterial cells are cocci. Cocci are always Gram positive or at least they should be.
- There are visible endospores. As stated above, this suggests, but does not prove, that the culture is Gram positive.

Colorblindness: If you're strongly protanopic like me, then telling the difference between purple and pink cells in a Gram stain can be difficult. Personally, I find it easier to distinguish the two colors in photographs of the stains (just hold your phone camera up to one of the microscope eyepieces. It takes some finesse, but you'll get it eventually). If you still can't tell, photo editing software such as GIMP (free and open source) can be used to analyze the color. Or just ask somebody without colorblindness to look at the stain for you. If you're feeling extra-ambitious, you can make an alternate counterstain using Bismarck Brown dye (see the References section). We actually have some in the media room.



SECTION 5 REFERENCES

Hucker, G. J. 1921. A new modification and application of the Gram Stain. J. Bacteriol. 6(4):395-397. <https://doi.org/10.1128/JB.6.4.395-397.1921>. **(Bismarck Brown alternate counterstain for the colorblind)**

Mangels, J. I., M. E. Cox, and L. H. Lindberg. 1984. Methanol fixation: an alternative to heat fixation of smears before staining. Diagn. Microbiol. Infect. Dis. 2(2):129-137. [https://doi.org/10.1016/0732-8893\(84\)90008-7](https://doi.org/10.1016/0732-8893(84)90008-7).

SECTION 6 METHOD VERSION & CHANGES

VERSION	DATE	EDITOR	COMMENTS
Version 1	2020-12-08	sjr267	Original SOP
Version 2			
Version 3			