The Microscopic Examination of Milk
Characterization of Milk Bacteria and Cells

The microscope has been used to observe and count bacteria and somatic cells in raw milk since the early 1900's. It has proven to be a valuable tool to the dairy industry. The milk smear procedure in use today is outlined in detail in the most recent edition of *Standard Methods for the Examination of Dairy Products* (i.e., SMEDP, 17th ed) and other references. For somatic cell counting, the Direct Microscopic Somatic Cell Count (DMSCC) is considered an official reference method used for regulatory purposes for direct milk counts and/or for calibration of approved electronic instruments. The regulatory procedure for somatic cells is outlined in detail in the most recent FDA 2400 Form. With the exception of the type of cells counted, the 2400 form procedure can be used for bacteria as well. While the Direct Microscopic Clump Count (DMCC) for bacteria is not considered an official test for bacteria counts, it is used throughout the dairy industry to estimate bacteria colony forming units (i.e., "clumps") in raw milk samples taken from the farm, the tank truck or the plant storage facility. The DMCC is most widely used to screen incoming raw milk supplies (i.e., tank-trucks) to determine whether the milk has an acceptable or legal bacterial load and has become accepted in some states as a legal method for rejection of unacceptable milk.

In addition to providing estimated counts of bacteria and somatic cells, the direct microscopic method has also been used as a trouble-shooting guide in attempts to identify the general types of bacteria present in a milk sample. Narrowing down the predominant types of contaminants in a sample can sometimes provide a lead as to the potential source or cause of a microbial defect. Guides with photographs of bacterial types were available as early as 1929 with a pamphlet develop by Dr. Breed, one of the originators of the DMC methods. Another brochure that is well known in the dairy industry in the northeast is the Vermont Extension Bulletin *Milk Under the Microscope,* which was developed by Atherton and Dodge in 1970. These brochures, which are no longer in print, presented pictures of bacterial types commonly seen in milk that were associated with potential inadequacies in the dairy farm production methods, including dirty equipment, poor cow hygiene procedures, mastitis and poor cooling. While these guides were useful, they were most effective when counts in the milk were high. It should also be point out that certain types of bacteria from very different sources can appear very similar under a direct microscopic smear resulting in guess work at best. In many cases however, it may be possible to narrow down the potential causes of high bacteria counts using the microscope as a tool.

The following pages present microscopic images and a summary of certain types of bacteria that are commonly associated with raw milk and dairy products. While pictures of these organisms as they appear with the DMCC are presented, caution must be used when using the microscope alone when trying to characterize the types of bacteria and other microorganisms associated with milk defects. Further information on these general groups of organisms is included with the photographs that would help the user further identify what they see under the microscope and perhaps on an agar plate. Auxiliary tests that might be used with colonies from an agar plate, including the Gram stain, spore stain, catalase test and oxidase test are also described. The groups discussed include:

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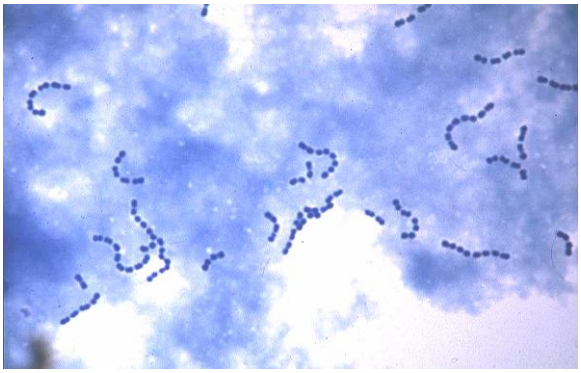


Fig. 1. Short chains of cocci from poor cooling and/ or dirty equipment. Mastitis source possible.

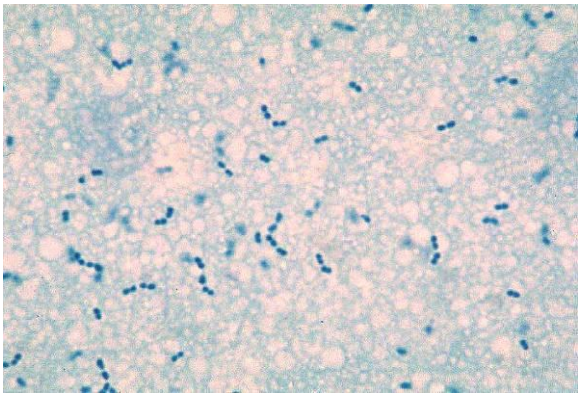


Fig. 2. Pairs of cocci from poor cooling and/or dirty equipment. Mastitis source possible.

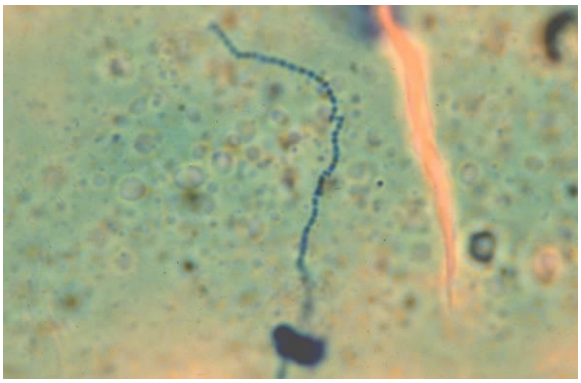


Fig. 3. Long chains of *S. agalactiae* as the result of mastitis infection, association with somatic cells.

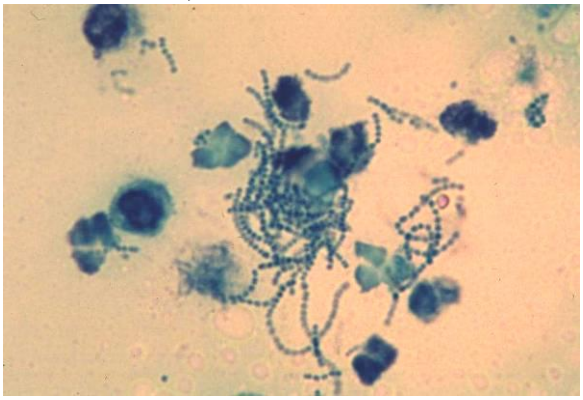


Fig. 4. Severe case of *S. agalactiae* infection from an individual cow showing close association with cells.

SPHERICAL COCCI; PAIRS OR CHAINS

When large numbers of pairs (diplococci) or short chains (streptococci) of spherical bacteria (cocci) are observed in raw milk, possible causes include poor cooling and/or dirty equipment. Environmental streptococci that cause mastitis may also appear as short chains or pairs while very long chains are typical of *Streptococcus agalactiae*. When mastitis is the cause, bacterial cells may be observed in association with somatic cells (leukocytes). With mastitis and poor cooling, milk smears may appear as mostly one type of bacteria, while high counts from dirty equipment would be more likely to contain a mixture of bacterial types (including rods, cocci in clusters).

Technical Information:

Gram-positive, cocci, 0.5-1.2 microns in diameter
Occur in pairs or chains of varying length
Catalase negative, oxidase usually negative
Colonies on SPC agar usually white, small, subsurface
Some strains survive pasteurization, most do not
Some strains may grow slowly under refrigeration

Streptococcus, ***Lactococcus*** and ***Enterococcus*** species are the most common Gram-positive, catalase-negative cocci that occur in milk. Cells are generally seen in pairs (diplococci) or in chains of varying lengths. They are easily recognized in milk smears; although distinguishing between specific types may be difficult.

Lactococcus (lactic streptococci) are involved in dairy fermentations (e.g., cheese) as well as in the spoilage of dairy products. **They are common in nature and the dairy environment and are often associated with plant materials including feeds and bedding materials. They may also thrive on milk soil of poorly cleaned equipment.** These organisms do not grow or grow slowly, under refrigeration, although they grow very well in milk at higher temperatures. **Poor cooling, especially when temperatures exceed 50-60°F, often results in proliferation of these organisms, seen in milk smears as pairs and/or chains of cocci. These organisms may be responsible for "sour" (high acidity) or "malty" defects in milk.**

Enterococcus (fecal streptococci) are often associated with fecal matter, although they survive well in other environments. They appear similar to *Lactococcus*. They may be associated with poor cooling, dirty equipment and in rare cases mastitis.

Streptococcus strains considered as common causes of mastitis include contagious strains (*S. agalactiae*), spread from cow to cow, and environmental strains (*S. uberis, S. dysgalactiae*) contracted from the environment (e.g., bedding). ***S. agalactiae*** **often appears in long chains, which may or may not, be seen associated with somatic cells. Other mastitis streptococci may be seen as pairs or chains of varying lengths resembling organisms in the above two figures (1 & 2).**

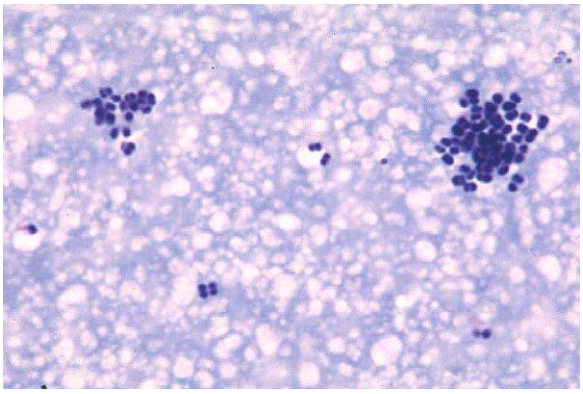


Fig. 5. *Micrococcus* in cluster formation associated with persistent soils on milk equipment.

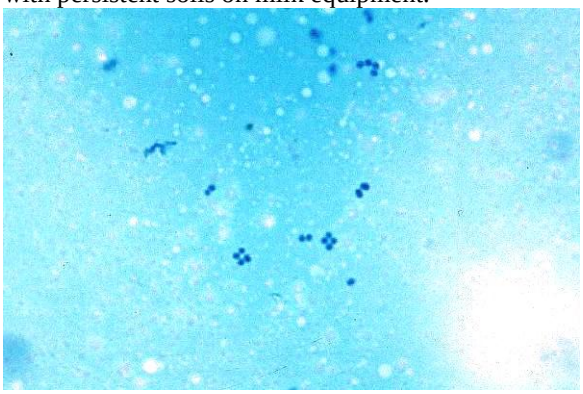


Fig. 6. *Micrococcus* in tetrad formation associated with persistent soils on milk equipment.

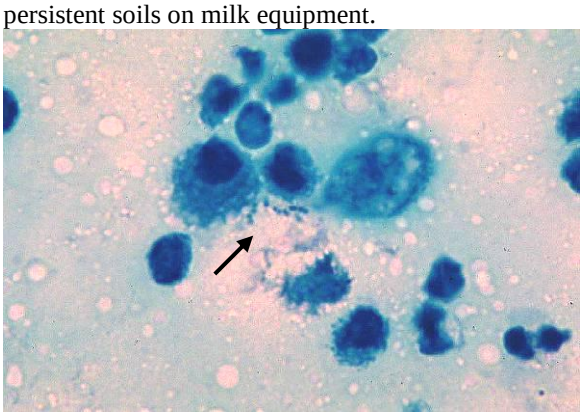


Fig. 7. *Staphylococcus aureus* associated with somatic cells in milk from an infected cow.

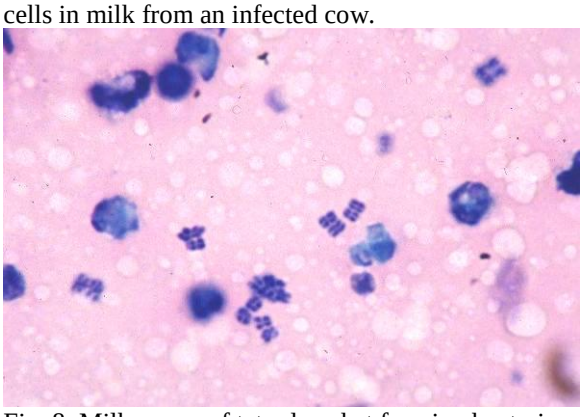


Fig. 8. Milk smear of tetrad packet forming bacteria associated with mastitis.

SPHERICAL COCCI; CLUSTERS, PAIRS OR TETRADS

Cocci in clusters (staphylococci) or in 2 x 2 arrangements (tetrads) are most often associated with persistent poor cleaning (e.g., milk stone, films) or in some cases mastitic cows. These organisms may also be seen as single cells or pairs, which may make it difficult to distinguish them from the streptococci group, although the cells may be larger. In general, they are rarely observed in raw milk as sole contaminants, or in high numbers, except perhaps when a cow with a *Staphylococcus* infection is shedding large numbers with its milk. The most common bacteria in this group include strains of ***Micrococcus*** and ***Staphylococcus*** (Staph.).

Technical Information - ***Micrococcus***:

Gram-positive cocci, 0.5-2.0 microns diameter
Occur as clusters, tetrads or pairs
Catalase positive, **oxidase positive**
Colonies on SPC usually opaque, white, yellow, orange
Some strains survive pasteurization
Some strains may grow slowly under refrigeration (rare)

Micrococcus spp. are common in milk as part of the natural flora of the cow, although they are generally present in low numbers. **Increased numbers of some strains have been associated with milk stone, cracked or old rubber parts and other areas of persistent poor cleaning. These strains are often thermoduric (i.e., survive pasteurization) and are generally not considered to be of the natural flora of the cow.** They may be present in the dairy environment, including bedding. *Micrococcus* spp. rarely reach significant numbers in raw milk. However, numbers may reach sufficient levels to influence fresh counts of pasteurized milk, although rarely above legal limits. Some strains may grow slowly in refrigerated milk.

Technical Information – ***Staphylococcus***:

Gram-positive, cocci, 0.5-2.0 microns diameter
Occur as single cells, pairs, clusters, tetrads
Catalase positive, **oxidase negative**
Colonies on SPC agar usually opaque, gray white
Generally do not survive pasteurization
Do not grow under refrigeration

Staphylococcus spp. are also commonly found in raw milk as part of the natural flora of the cow, although generally in low numbers. **Strains of *Staph. aureus* are associated with contagious mastitis and may be shed into milk, although the influence on bulk tank counts is not as common as with mastitic streptococci species.** Other *Staph*. species also cause mastitis. They may be present in the dairy environment, including bedding, although most strains are associated with the skin of the cow and perhaps dairy personnel. **The potential for growing on soiled equipment exists**, but this has not been well documented. **Most strains are not considered thermoduric or psychrotrophic.**

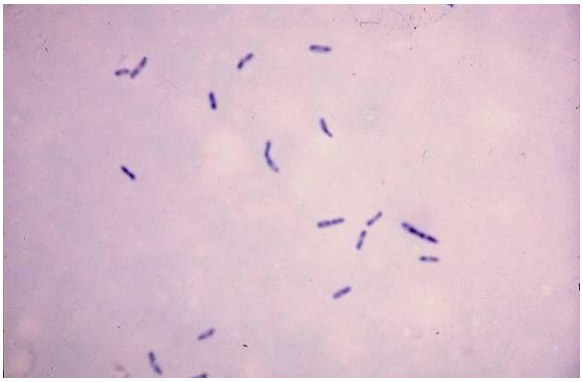


Fig. 9. Pseudomonas strain from raw milk stored at marginal refrigeration for extended period.

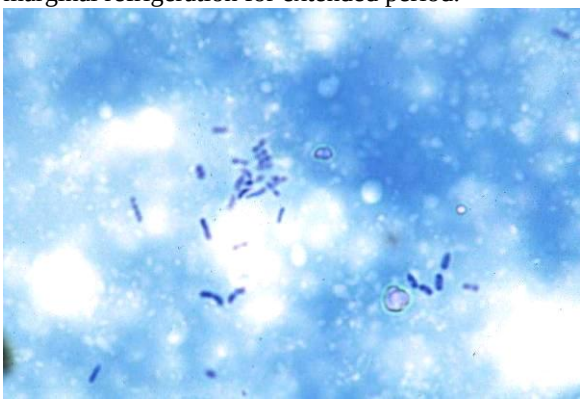


Fig. 10. Coliform bacteria from raw milk, this strain is a smaller rod. Coliform rods will vary in size.

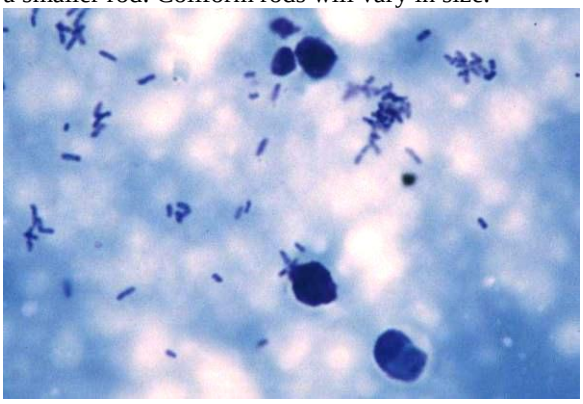


Fig. 11. Coliform bacteria from a mastitic cow with elevated SCC and clinical signs.

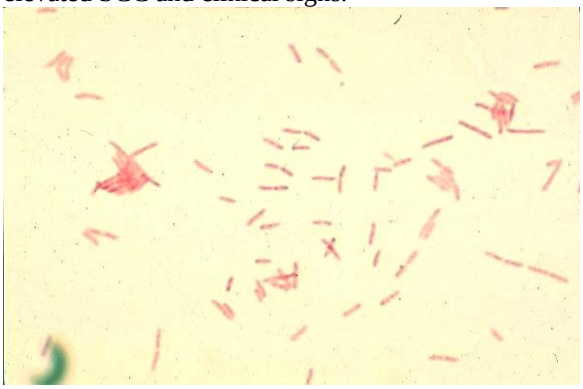


Fig. 12. **Gram-stain** of *Pseudomonas* spp. isolated from raw milk. Cells stain red with Gram-stain.

SHORT - MEDIUM RODS; SINGLES, PAIRS

Rod shaped bacteria (bacilli) are common in raw milk, although it is often difficult to distinguish between types. **Those types most often associated with high counts in raw milk are primarily Gram-negative rods and include psychrotrophs (e.g., *Pseudomonas*) and coliform bacteria**. **In milk smears, these appear as short to medium rods, growing singly or in pairs.** Some strains exhibit bi-polar staining (stain darker on the ends). **These organisms in raw milk are most often associated with contamination due to poor cleaning and sanitation procedures as well as environmental sources and poor pre-milking hygiene. Marginal cooling may cause psychrotrophic strains to increase.**

Gram-negative bacteria are widespread in nature. *Pseudomonas* spp. are often associated with untreated water supplies, although they are common in the environment and will grow well on soiled milking equipment. Coliform bacteria and Gram-negative bacteria in general, have been found in high numbers in bedding materials. Poor pre-milking hygiene procedures may be a source of these organisms although high counts directly from soiled teats would be rare. While coliforms are often associated with manure, some strains thrive in the environment and on poorly cleaned equipment as do other Gram-negatives. **Counts in raw milk resulting from soiled and/or poorly sanitized equipment may be low. However, *Pseudomonas* strains that contaminate milk from this source are most often responsible for high Preliminary Incubation (PI) counts.** Extended refrigeration selects for psychrotrophic bacteria, which often dominate raw milk at the plant.

Certain coliform bacteria and other Gram-negative rods are common causes of mastitis and may be shed into the milk from infected cows, although this is not as common as with mastitic streptococci. Certain strains of *Pseudomonas* may cause mastitis, but these are relatively rare and the influence of shedding into the milk has not been well documented.

Technical Information – ***Pseudomonas,*** *related bacteria*:

Gram-negative rods, 0.5-1.0 by 1.5 – 5.0 microns
Occur singly or in pairs, some strains stain bi-polar
Catalase positive, **oxidase-positive**
Colonies on SPC usually translucent, various pigments
- Large circular surface colonies are common
- Some strains produce diffusible pigments (green)

Do not survive pasteurization
Most common psychrotroph

Technical Information – ***Coliform,*** *related bacteria*:

Gram-negative rods, 0.5-1.0 by 1.5 – 5.0 microns
Occur singly or in pairs, some strains stain bi-polar
Catalase positive, **oxidase negative**
Colonies on SPC usually translucent, often mucoid
- Large surface colonies are common

Colonies on Coliform media (VRBA) are dark red
Do not survive pasteurization
Some strains are psychrotrophic

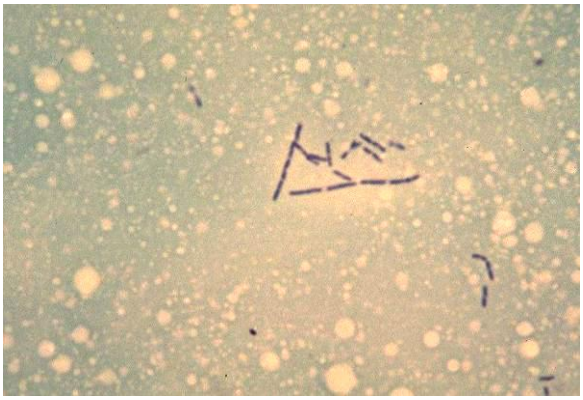


Fig 13. Milk smear of Gram-positive *Bacillus* strain demonstrating similarities to Gram-negative rods.

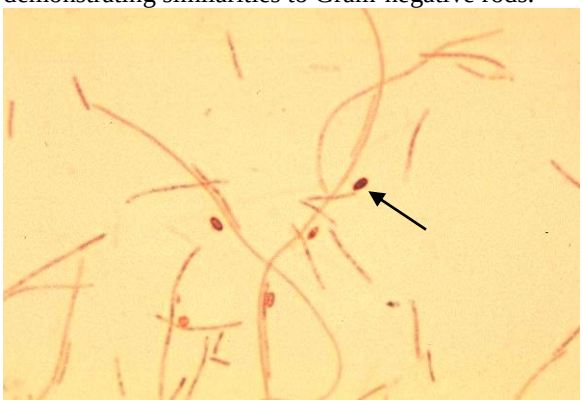


Fig. 14. **Spore Stain** of Gram-positive *Bacillus* strain demonstrating vegetative growth and spores.

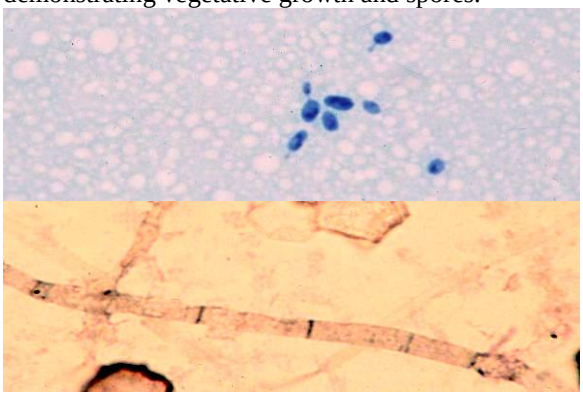


Fig. 15. Milk smear of budding yeast (top) and mold filament (bottom) from aged milk soil.

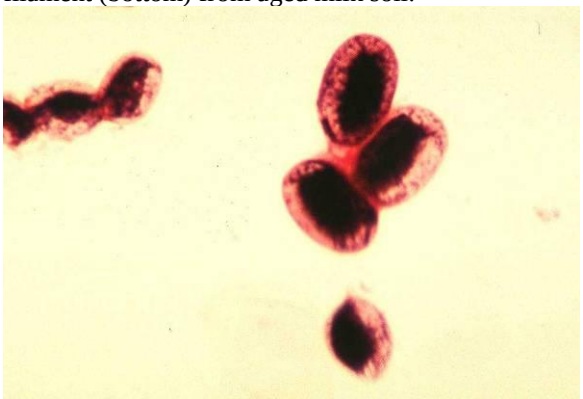


Fig 16. **Gram-stain** of *Prototheca* isolated from milk of a cow with mastitis.

GRAM-POSITIVE RODS; VARIOUS LENGTHS AND SIZES

Gram-positive rod shaped bacteria generally do not occur at high numbers in raw milk although a few strains may be significant contaminants. *Bacillus*, *Paenibacillus*, *Microbacterium* and *Lactobacillus* are the most common. These organisms are common in the dairy environment, although they generally do not grow as rapidly in milk as the previously discussed groups. *Bacillus* and *Paenibacillus* spp. are found more frequently in both raw and pasteurized milk. These are spore-formers, most of which survive pasteurization. Some strains of Gram-positive rods are psychrotrophic, especially the spore formers, although they do not grow as quickly as Gram-negative psychrotrophs that do not survive pasteurization. However, they do have the potential to become significant spoilage organisms when post-pasteurization contamination is prevented. These tend to be hardy organisms that are more likely to survive during cleaning and sanitation procedures and other extreme conditions (e.g., drying).

Technical Information – ***Gram-positive Rods***:

Gram-positive rods, various widths, lengths
Occur singly, in pairs or as end-to-end chains
Rods of some strains have blunt or squared ends
Catalase, oxidase, colony morphology varies
Some strains produce spores (i.e., *Bacillus*, *Paenibacillus*)
Some strains survive pasteurization
Some strains are psychrotrophic

YEAST AND MOLDS

Yeast and molds generally do not occur in large numbers in raw milk though if milk soil remains within uncleanable areas of the milking system, these organisms, especially yeasts, may become significant. Some yeast may be involved in mastitis though this is relatively rare. Yeast, which are much larger than most bacteria, are spherical to oval and generally exhibit budding reproduction. Molds most often appear as large filaments. Both yeast and molds will grow on SPC agar though growth rates are generally slower for most molds. Both types of organisms are common in nature.

PROTOTHECA

***Prototheca* spp. are achlorophyllic (do not contain chlorophyll) algae (microscopic plants) that can cause mastitis in dairy animals.** Primary sources include soil, plants, water (esp. stagnant) and feces. This organism appears as large spherical cells that may be easily mistaken for somatic cells. They also grow on SPC agar and have been responsible for high counts in milk.

AUXILIARY TESTS FOR BACTERIAL CHARACTERIZATION FROM AGAR PLATES

The microscopic examination of raw milk is often used to trouble-shoot high bacteria counts. While milk smears may provide evidence of microorganisms associated with poor cooling and mastitis, other causes of high counts may not be as clear. In many cases, further characterization is required. Generally this requires isolation of the suspect organism(s) from an agar plate (see p. 8 for isolation-streaking procedure). The Gram-stain is one of the first procedures used when classifying bacteria. Other tests include the endospore stain, the catalase test and the oxidase test. The results of these tests are listed under the technical information in the groups of bacteria previously described.

STAINING PROCEDURES FOR COLONIES ON AGAR PLATES

A. Making a Smear from Agar Plates (for isolation/streaking procedure, see p. 8):

1. Flame sterilize transfer loop and cool on a clear sterile area of the agar plate or Petrifilm™. Alternatively, use sterile disposable inoculating loops.
2. Touch desired colony with the loop or needle picking up a small amount of microbial growth. Thoroughly mix growth with a drop of sterile buffered water on a slide to form a thin film.
3. Allow the film on the slide to air dry, then heat fix the slide by passing three times through the flame. The slide should feel warm, but not hot, when placed against the back of your hand. Do Not Over-Heat.
4. Stain the smear with: Levowitz-Weber or Simple Stain (Gram Crystal Violet)
   Gram-Stain Procedure, Endospore Stain Procedure
5. Blot the slide dry and observe under the microscope (oil immersion lens, 100x).

B. Gram Stain Procedure:

Reagents for the Gram stain include crystal violet, Gram's iodine, decolorizer, and safranin. Prepared kits with directions for use are available from laboratory supply companies. General procedure:

1. Prepare a thin smear of the organism, allow to air dry, and then fix with heat. **Ideally the culture should be 18 - 24 hr. old**. Smear may be made from plate or broth cultures (e.g., nutrient broth).
2. Cover smear with crystal violet solution for 1 minute then rinse gently with water.
3. Cover smear with Gram iodine solution for 1 minute or longer. Rinse gently with water.
4. Apply decolorizer solution just until it runs clear (no more color, ~ 20 seconds). Quickly rinse off remaining decolorizer with water. **DO NOT OVER-DECOLORIZE**.
5. Remove excess water. Counter-stain with safranin solution for 1 minute. Rinse with water and blot dry without rubbing.
6. Examine cells microscopically: Cells Stain **Blue = Gram-positive**
   Cells Stain **Red = Gram-negative**

C. Potassium Hydroxide (KOH) Method for Gram Reaction:

1. Place one drop of 3 % KOH on a clean glass slide.
2. Remove bacterial growth from a colony with an applicator stick or sterile loop.
3. Mix growth with KOH and pull away slowly.
4. **Gram-negative will be ropy or thread-like. Gram-positive will not.**

D. Endospore (Spore) Stain (cold method): Older cultures tend to have more spores present, strain dependent. Some require special media to form spores. Spores may be observed free or within cells.

1. Prepare a thin smear of the organism in question, allow to air dry thoroughly. **Fix the smear by passing through a flame 20 times.**
2. Flood the smear with **7.6 % malachite green** (aqueous solution, 7.6. g/100 ml water) for 15-30 minutes (30 minutes may be needed).
3. Rinse gently with water until clear. Counter-stain with **safranin** for 30 seconds. Rinse, blot dry.
4. **Endospores stain green, while the remainder of the cell stains light red**.

BIOCHEMICAL TESTS USED TO CHARACTERIZE BACTERIA

A. Catalase Test:

The catalase test determines if an organism can degrade peroxides (i.e., H_2O_2) to oxygen and water. The enzyme catalase is present in certain bacteria as a protective feature to destroy toxic peroxides. When hydrogen peroxide is added to a culture of bacteria that has the catalase enzyme, visible bubbles of oxygen are liberated during its degradation. Most Gram-negative bacteria common in dairy products are catalase-positive. The catalase test is most useful in distinguishing between certain Gram-positive bacteria (catalase-positive *Micrococcus* versus catalase-negative *Streptococcus* or *Lactococcus*).

Procedure:

1. From an agar plate, transfer a small amount of a colony to the surface of a clean dry slide with a sterile loop or applicator stick. Alternatively, the test can be done directly on a colony.
2. Add one drop of 3 - 5 % Hydrogen Peroxide.
3. **Visible bubbles indicate a positive catalase test**. No bubbles indicate a negative catalase test. Certain strains are weakly positive; a low-powered microscope may be required.

   Catalase-positive: visible bubbles with hydrogen peroxide
   Catalase-negative: no bubbles formed with hydrogen peroxide

B. Oxidase Test:

The oxidase test determines the presence of the enzyme cytochrome-c oxidase (important in cell respiration or electron transport) and is used primarily to distinguish between different groups of Gram-negative bacteria. Members of the family Enterobacteriaceae, which includes the coliform group, *Salmonella*, *Shigella*, and *Proteus*, are oxidase-negative. Gram-negative bacteria that are oxidase-positive include the most commonly occurring psychrotrophs in milk belonging to the genus *Pseudomonas*. *Alcaligenes* and *Flavobacterium* are other genera that are oxidase-positive. The oxidase test is also used to differentiate *Micrococcus* (ox-pos) from *Staphylococcus* (ox-neg).

Procedure - Liquid Reagent:

1. Prepare the "oxidase" reagent just before use. Weigh 0.1 gram of para-aminodimethylaniline oxalate and dissolve in 10 ml of distilled water with gentle heating. This reagent is available prepared in sealed vials.
2. Soak an area of filter paper with the "oxidase reagent."
3. Using a wooden applicator stick, toothpick, or platinum loop (do not use standard loop material), apply a portion of bacterial growth from an isolated colony to the moistened area.
4. **If the organism is oxidase-positive the reagent will turn the growth red to black within 2 minutes**. If they are oxidase-negative, no color will develop.

Procedure - Dryslide™ OXIDASE (BBL):

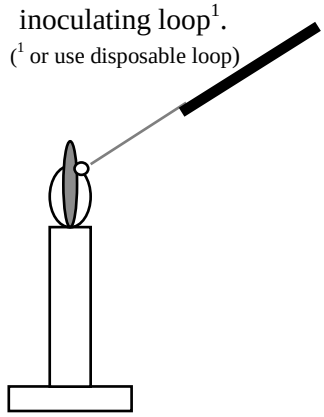
1. Open pouch and remove the number Dryslides to be used. Seal pouch tightly, store at room temperature and use the remainder within one week.
2. Using a wooden applicator stick, toothpick, or platinum loop, apply a portion of bacterial growth from an isolated colony to the reaction area of the slide.
3. **Oxidase-positive, the slide will turn purple within 20 seconds**. If it is oxidase-negative, no color will develop. Disregard color changes after 20 seconds.

References:
1. National Mastitis Council. 1999. *Laboratory Handbook on Bovine Mastitis*. NMC, Madison, WI.
2. Murphy, S.C. and K.J. Boor. 2000. Trouble-shooting sources and causes of high bacteria counts in raw milk. Dairy, Food and Environmental Sanitation. 8:606-611.
3. Hayes, M.C., R.D. Ralyea, S.C. Murphy, N.R. Carey, J.M. Scarlett and K.J. Boor. 2001. Characterization of elevated microbial counts in bulk tank raw milk. J. Dairy Sci. 84:292-298.
4. Marshall, R.T. editor. 1993. *Standard Methods for the Examination of Dairy Products*. 16th Edition APHA, Washington D.C.
5. Atherton, H.V. and W.A. Dodge. 1970. *Milk Under the Microscope*. Vermont Extension Serv, Univ. of Vt.

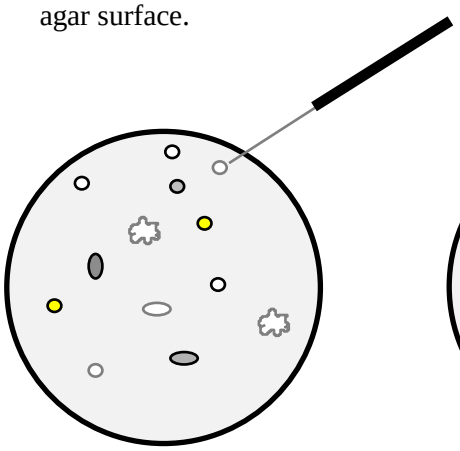
Appendix

Streaking Isolated Bacterial Colonies for Purification and Identification

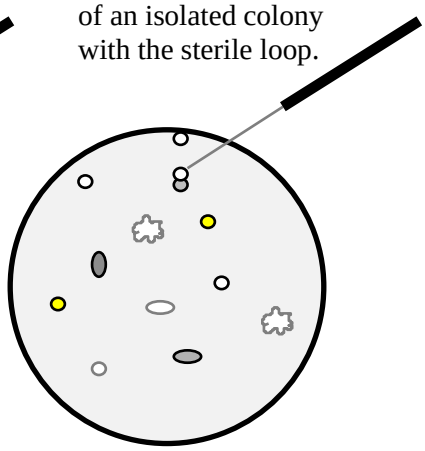
1. Flame sterilize inoculating loop[1].
([1] or use disposable loop)



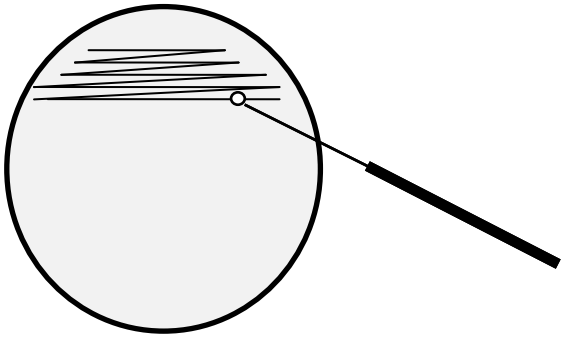
2. Cool loop on sterile agar surface.



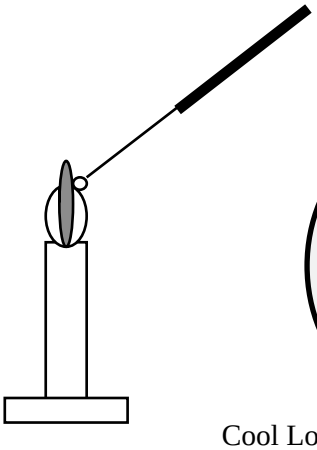
3. Pick up a small amount of an isolated colony with the sterile loop.



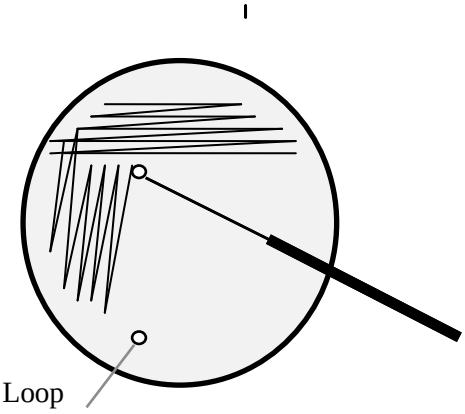
4. Streak the loop across a section of a pre-poured sterile SPC agar plate, back and forth several times over the surface of the plate.



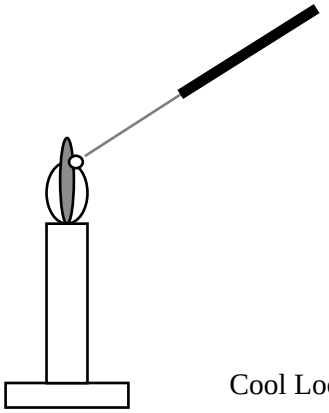
5. Flame sterilize the loop[1], and cool on a sterile section of the agar surface.



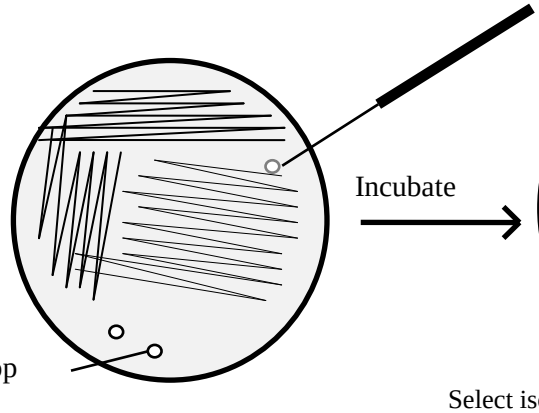
6. Streak into the previous streaked section once or twice, continue streaking across one third of plate



7. Flame sterilize the loop[1] and cool on plate.



8. Streak into previous section once or twice, continue streaking final third of plate.



9. Incubate. Select isolated colonies for further identification.