Lab 7 - Microbial and Fungal Diversity

Laboratory Objectives:

- Describe bacterial structure: colony morphology, cell shape and state of aggregation.
- Describe the results of Gram staining and discuss the implications to cell wall chemistry.
- Describe a scenario for succession of bacterial and fungal communities in aging milk, relating this to changes in environmental conditions such as pH and nutrient availability.
- Become familiar with other types of fungi and their interactions with other organisms.
- To enjoy some of the benefits of bacterial and fungal growth and metabolism.

Textbook Reference Pages: pp. 560-579 and 650-667

Part 1 – Microbial Ecology

(parts adopted from *Investigating Biology Lab Manual*, 5th edition, 2005, Pearson Education, Inc.)

A. Introduction

In this lab exercise, you will study organisms commonly called **bacteria**. In the five-kingdom scheme, bacteria were placed in the kingdom **Monera**. In the three-domain system, the common bacteria are classified in the domain **Bacteria**. Bacteria are small, relatively simple, prokaryotic, single-celled organisms. **Prokaryotes**, from the Greek for "prenucleus," have existed on Earth longer and are more widely distributed than any other organismal group. Prokaryotes include the Archaea and they are found in almost every imaginable habitat: air, soil, and water, in extreme temperatures and harsh chemical environments. They can be photosynthetic, using light, or chemosynthetic, using inorganic chemicals as the source of energy, but most are heterotrophic, absorbing nutrients from the surrounding environment.

Most bacteria have a **cell wall**, a complex layer outside the cell (plasma) membrane. The most common component found in the cell wall of Bacteria is **peptidoglycan**, a complex protein-carbohydrate polymer. There are no membrane-bound organelles in bacteria and the genetic material is not bound by a nuclear envelope. Bacteria do not have chromosomes; their genetic material is a single circular molecule of DNA. In addition, bacteria may have smaller rings of DNA called **plasmids**, consisting of only a few genes.

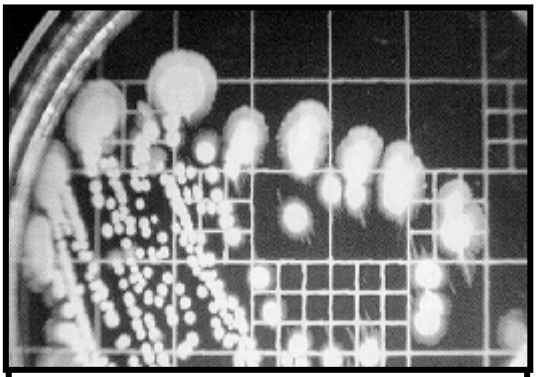


Figure 1: *Bacillus subtilis*, a rod-shaped bacterium, has colonies that grow with an opaque center and spreading edge (from Leboffe & Pierce, 1999).

The microbiological scale of inquiry.

Because our unaided eyes cannot discern objects smaller than about one-hundredth of a millimeter (10 microns), we cannot see a major portion of the living world. To help remedy this, we need to extend our vision to a much smaller scale. One obvious tool is the microscope. A less obvious tool involves culturing techniques, which allow the activities in the microscopic world to proceed under carefully controlled conditions until they yield something that can be seen with the naked eye. Using the latter approach and agar plates, you will sample the microbial populations of milk in this lab.

Colony Morphology. Bacteria reproduce by a process called **binary fission**, in which the cell duplicates its components and divides into two cells. These cells usually become independent, but they may remain attached in linear chains or grapelike clusters. In favorable environments (such as growth on solid substrates), an individual bacterial cell rapidly proliferates to form a **colony,** which is composed of millions of identical cells. By allowing an individual bacterium to divide and multiply into a colony, you will be able to see population characteristics with the naked eye.

Colonies of different bacteria look different (Figs. 1-3). Each colony has a characteristic size, shape, consistency, texture, and color (colony morphology), all of which may be useful in preliminary species identification. Thus, observation of differences in colony morphology will provide us with our first clues about the identity of the bacteria on the agar plates. Use Figure 4 to become familiar with this terminology.

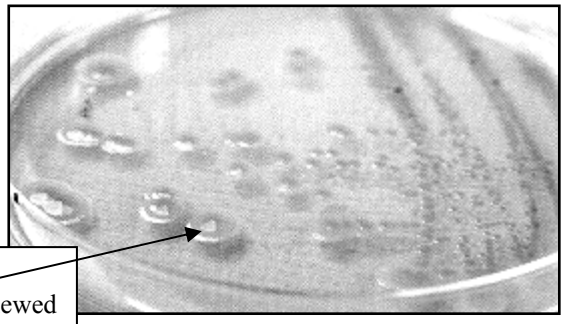


Figure 2: Colonies of *Serratia marcescens*, gram-negative rod-shaped bacteria, appear shiny and almost cone shaped if viewed at an angle with a strong light source (from Leboffe & Pierce, 1999).

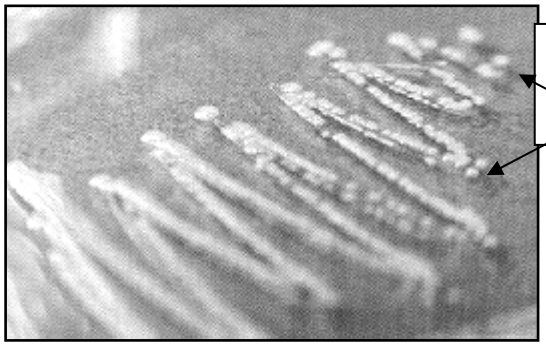
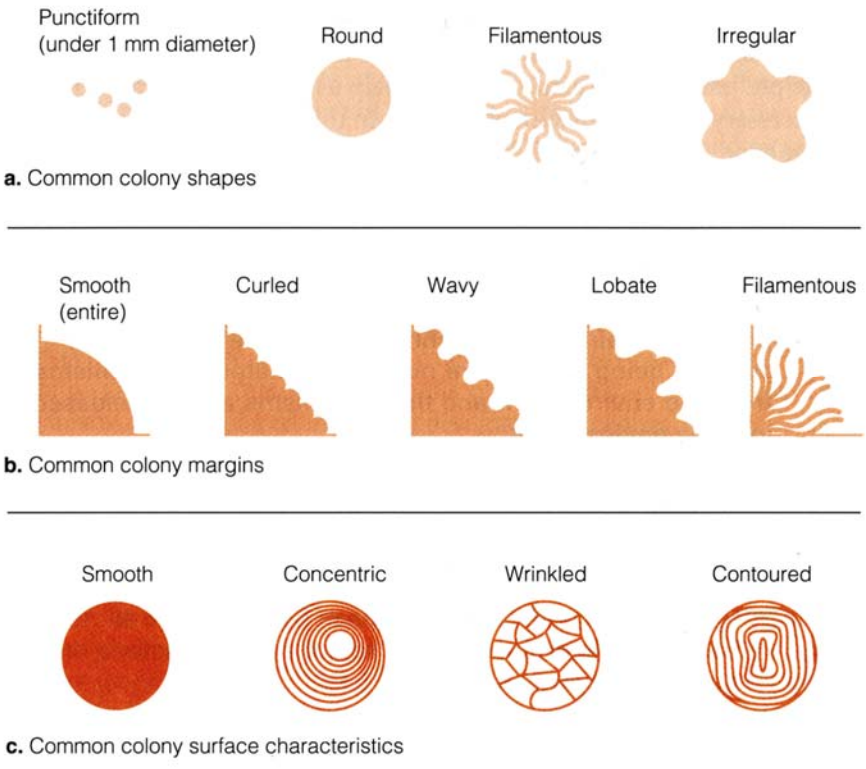


Figure 3: This gram-negative, rod shaped bacteria (*Klebsiella pneumoniae*) grows into relatively dull and rounded (convex) colonies with a smooth edge (from Leboffe & Pierce, 1999).

Figure 4. Terminology used in describing bacterial colonies. (a) Common shapes, (b) margins, and (c) surface characteristics are illustrated.



Occasionally, one or more fungal colonies may contaminate the bacterial plates. Some fungi can be distinguished from bacteria by the fuzzy appearance of the colony (Figure 5). Elsewhere in lab today, you will see that the body of such a fungus is a mass of filaments called **hyphae** in a network called a **mycelium**. Learn to distinguish fungi from bacterial colonies.

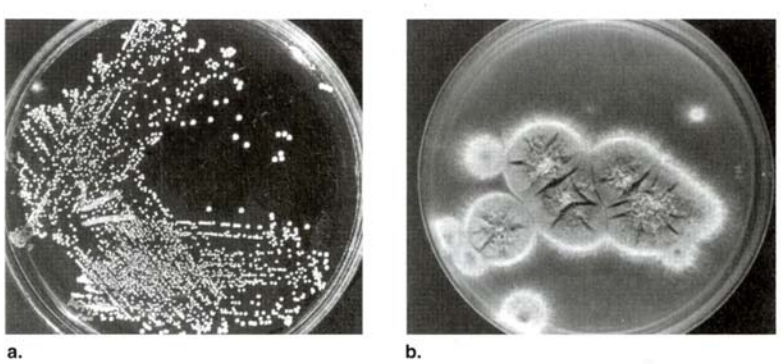


Figure 5.
(a) Bacteria and (b) fungi growing on nutrient agar plates. The body of most fungi consists of filaments called *hyphae* in a network called a *mycelium.* The hyphae give fungal colonies a fuzzy appearance.

By changing the ingredients in the growth media we can create selective media, which inhibit the growth of some kinds of bacteria and facilitate the growth of others. The ability of a microorganism to grow on various types of media therefore provides us with clues about its identity. Students in the Biology 19 and Biology 24 labs use selective media to isolate and characterize a variety of yeast mutants.

Morphology of Individual Cells. In addition to differences in colony morphology, the shape of individual bacterial cells is important in distinguishing characteristics of bacterial species. Because of the small size and similarity of cell structure in bacteria, techniques used to identify bacteria are different from those used to identify macroscopic organisms. Staining reactions and properties of growth, nutrition, and physiology are usually used to make final identification of species, many of which are beyond the scope of this lab. However, the structure and arrangement of cells contribute preliminary information that could help us determine the appropriate test to use to make a definitive identification.

Microscopic examination of bacterial cells reveals that most bacteria can be classified according to three basic shapes: **cocci** (spheres), **bacilli** (rods), and **spirilla** (spirals, or corkscrews). In many species, cells tend to adhere to each other and form aggregates, with each cell maintaining its independence. Simple staining procedures, such as using the dye methylene blue, allow one to distinguish *Streptococcus* or *Staphylococcus* from each other and from other bacteria. *Streptococci* are spherical bacteria that grow end-to-end, forming bacterial chains (Fig. 6); they are a common cause of severe sore throats and severe skin infections such as impetigo.

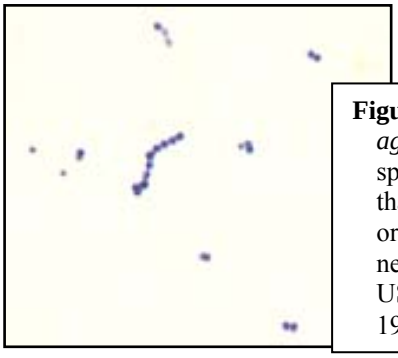


Figure 6: *Streptococcus agalactiae* is gram-positive spherical (coccus) bacteria that is usually seen in pairs or chains. Major cause of neonatal meningitis in the US (from Leboffe & Pierce, 1999).

In contrast, *Staphylococci* are spherical bacteria that attach to one another to form grape-like clusters (Fig. 7). Common species grow on the skin without causing any harm, but some

species can cause severe infections, with boils or impetigo. *Staphylococcus aureus* can also cause severe respiratory tract diseases such as pneumonia.

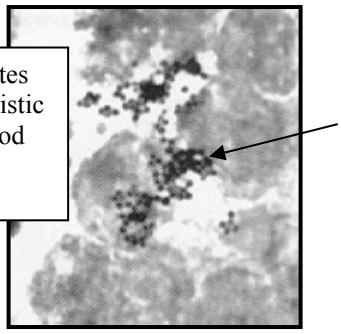


Figure 7: Gram staining of *Staphylococcus aureus* indicates this is a gram positive bacterium that grows in characteristic grape-like clusters. This bacterium is responsible for food poisoning, toxic shock syndrome, and abscesses almost anywhere in the body (from Leboffe & Pierce, 1999).

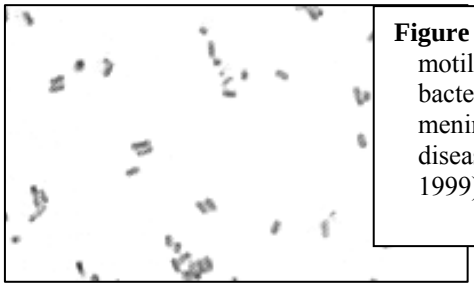


Figure 8: *Escherichia coli* is a motile, gram-negative, rod-shaped bacteria, responsible for meningitis and various diarrheal diseases (from Leboffe & Pierce, 1999).

An example of a bacillus bacterium is *Escherichia coli*, a well-characterized species used in research labs around the world. *E. coli* is a common inhabitant of the intestines of healthy people, but can cause irritating infections of the urinary tract, and life threatening infections of the intestinal tract, especially among infants or people with compromised immune systems (Fig. 8). Recent outbreaks of life-threatening disease associated with fast-food items have been attributable to contamination by *Escherichia coli*.

The bacterium that causes syphilis (*Treponema pallidum*) is also gram negative, but is spiral-shaped (Figure 9).

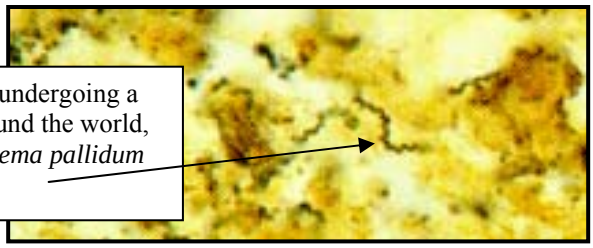


Figure 9: Syphilis, a disease that is undergoing a resurgence in this country and around the world, is caused by the bacterium *Treponema pallidum* (from Leboffe & Pierce, 1999).

Examine the poster on display in lab as well as the slides on the demonstration microscopes for more examples of bacterial morphology.

When working with bacteria, it is very important to make sure that the cultures being studied are not contaminated by organisms from the environment and that organisms are not released into the environment.

1. Wash your hands before and after performing an experiment.
2. Using the alcohol lamp or Bunsen burner, flame all inoculation loops used to manipulate bacteria or fungi before and after use. ***Never place one of these items on the lab bench after use without flaming it!***
3. Wear a lab coat, a lab apron, or a clean old shirt over your clothes to lessen chances of staining or contamination accidents.
4. Use safety glasses when performing the Gram stain and keep the bottles away from the Bunsen burner flame, as the chemicals cause eye irritation and are flammable, respectively.

The bacteria used in these exercises are not pathogenic (disease-producing); nevertheless, use appropriate techniques and work with care! If a spill occurs, wear disposable gloves, and wipe up the spill with paper towels. Follow this by washing the affected area with soap and water and a disinfectant. ***Dispose of the gloves and soiled towels in the yellow trash can.***

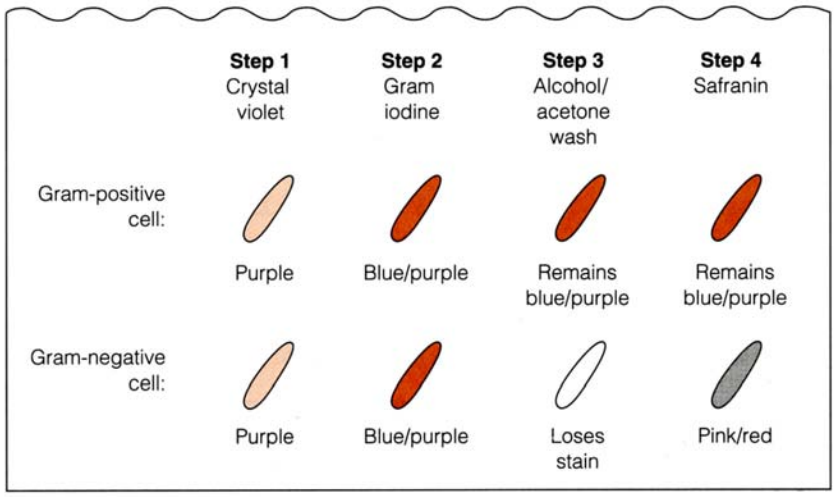
Identifying Bacteria by the Gram Stain Procedure. The gram stain is commonly used to assist in bacterial identification. This stain, first developed in 1884, separates bacteria into groups, depending on their reaction to this stain. Bacteria react by testing either gram-positive, gram-negative, or gram-variable, with the first two groups being the most common. Although the exact mechanisms are not completely understood, scientists know that the response of cells to the stain is due to differences in the complexity and chemistry of the bacterial cell wall. Recall that bacterial cell walls contain a complex polymer, peptidoglycan. The cell walls of gram-negative bacteria contain less peptidoglycan than gram-positive bacteria. In addition, cell walls of gram-negative bacteria are more complex, containing various polysaccharides, proteins, and lipids not found in gram-positive bacteria. Gram-staining properties play an important role in bacterial classification.

Use the following procedure to discover whether your different colonies of bacteria are comprised of gram-positive or gram-negative cells.

1. Place a small drop of water in the center of a clean microscope slide.
2. Select a bacterial colony by flaming the inoculating loop and cool the loop by touching it to a clear area of agar. Then, gently scrape the colony off the surface of the agar.
3. Spread the bacterial suspension in the drop of water over a one-inch area.
4. Let the smear air dry. Then heat-fix the smear by passing the slide through the low flame of a Bunsen burner three times (with the bacterial smear facing away from the flame).
5. Allow the slide to cool. Then, place a few drops of crystal violet solution on the smear.
6. After one minute, *gently* rinse the slide with deionized water (from the squirt bottle).
7. Shake off the excess water and cover the smear with iodine solution to set the stain.
8. After one minute, *gently* rinse the slide with deionized water (from the squirt bottle).
9. Rinse the slide gently with decolorizer for approximately 10 seconds. Decolorization is complete when the solution runs clear from the slide. **DO NOT OVERDO THIS STEP!**
10. *Immediately* rinse the slide with deionized water to prevent further destaining.
11. Shake off excess water and apply the safranin counter stain (which stains the gram negative bacteria a pink/red color; the gram-positive bacteria will be unaffected by the safranin).
12. Rinse the slide gently with deionized water (from the squirt bottle), blot dry, and examine with the compound microscope and the 40X objective.
13. Compare your slide with slides prepared by other students to allow you to see the difference between gram-negative and gram-positive bacteria.

Thus, gram staining relies on the use of three stains: crystal violet (purple), Gram iodine (brown/black), and safranin (pink/red). Gram-positive bacteria (with the thicker peptidoglycan layer) retain the crystal violet/iodine stain and appear blue/purple. Gram-negative bacteria lose the blue/purple stain but retain the safranin and appear pink/red (Figure 10).

Figure 10.
The Gram stain. Crystal violet and Gram iodine stain all cells blue/purple. Alcohol/acetone destains gram-negative cells. Safranin stains gram-negative cells pink/red.



In summary:

Gram-Negative Bacteria	**Gram-Positive Bacteria**
more complex cell wall	simple cell wall
thin peptidoglycan cell wall layer	thick peptidoglycan cell wall layer
outer lipopolysaccharide wall layer	no outer lipopolysaccharide wall layer
retain safranin	retain crystal violet/iodine
appear pink/red	appear blue/purple

B. Ecological Succession of Bacteria in Milk

(lab adopted from *Investigating Biology Lab Manual*, 5th edition, J. G. Morgan and M. E. B. Carter, 2005 Pearson Education, Inc.)

Materials

pH paper
flasks of plain and chocolate whole milk: refrigerated and aged 1, 4, and 8 days
TGY agar plates of each of the milk types
supplies for Gram stains

Introduction

Milk is a highly nutritious food containing carbohydrates (lactose, or milk sugar), proteins (casein, or curd), and lipids (butterfat). This high level of nutrition makes milk an excellent medium for the growth of bacteria. Pasteurizing milk does not sterilize it (sterilizing kills all bacteria) but merely destroys pathogenic bacteria, leaving many bacteria that will multiply very slowly at refrigerated temperatures; but at room temperature, these bacteria will begin to grow and bring about milk spoilage. Biologists have discovered that as milk ages, changing conditions in the milk bring about a predictable, orderly succession of microorganism communities (associations of species).

Community succession is a phenomenon observed in the organizational hierarchy of all living organisms, from bacterial communities in milk to animal and plant communities in a maturing deciduous forest. In each example, as one community grows, it modifies the environment, and a different community develops as a result.

In this laboratory exercise, you will work in pairs and observe successional patterns in two types of milk, plain whole milk and milk with sucrose and chocolate added. You will record changes in the environmental conditions of the two types of milk as they age. Note certain observations scientists have made about milk bacteria and their environment.

1. *Lactobacillus* (gram-positive rod) and *Streptococcus* (gram-positive coccus) survive pasteurization.
2. *Lactobacillus* and *Streptococcus* ferment lactose to lactate and acetic acid.
3. An acidic environment causes casein to solidify, or curd.
4. Two bacteria commonly found in soil and water, *Pseudomonas* and *Achromobacter* (both gram-negative rods), digest butterfats and give milk a putrid smell.
5. Yeasts and molds (both fungi) grow well in acidic environments.

Scenario

Propose a scenario (the hypothesis) for bacterial succession in each type of milk.

On the front lab bench are four flasks of plain whole milk and four flasks of chocolate milk. One flask of each has been kept under refrigeration. One flask of each has been at room temperature for 24 hours, one for 4 days, and one for 8 days. On the front bench there are also TGY (tryptone, glucose, yeast) agar plate cultures of each of the types of milk.

One team of two students should work with plain milk of each type, another with chocolate milk of each type. Teams will then exchange observations and results.

Procedure

1. Observe and describe the bacterial/fungal colonies for the agar plate(s) that you were given. Use the vocabulary in Figure 4 and on the bottom of the plate worksheet on the next page, on which you should also record your raw data. (One worksheet should be completed for each plate analyzed.)
2. Calculate the Shannon-Weiner Index of Diversity for each plate, as described below. Record the result on your plate worksheet.
3. Prepare Gram stains for each different **type** of bacterial colony on each plate using the staining instructions above.
4. Record the results of the Gram stains on your plate worksheet and in Table 1 on page 10.
5. Using the pH paper provided, take the pH of your flask. Record your results in Table 1.
6. Record the odor (sour, putrid), color, and consistency (coagulation slight, moderate, chunky) of the milk in each flask. Record your results in Table 1.
7. Record a summary of your individual results in Table 1, then transcribe your results to the lab overhead of Table 1. Use the results of your classmates to complete Table 1, which describes the characteristics of each milk culture and the microbes present in each.
8. Answer the Discussion questions on p. 11.
9. **Turn in a worksheet for each plate (p. 9) and pp. 10 -11 at the beginning of lab next week**.

Calculation of Diversity

A simple tally of the number of colonies fails to incorporate some key information, such as the composition of species. To get a sense of how the microbial communities in milk change as a function of time at room temperature, we can calculate the species diversity, which measures both the number of kinds and the evenness of individuals among those kinds. We will use the Shannon-Wiener index of diversity, H':

$$H' = -\sum_{i=1}^{S} p_i \ln p_i,$$ where S is the total number of types

The symbol p_i represents the proportional abundance of that kind of colony. So, if you had 80 colonies and 20 of them were of the same type, p_i for that type would be 0.25 (i.e., 20/80). The quantity $p_i \ln p_i$ for that type would be (0.25)(-1.39) = -0.35. To calculate H', the analogous values would be calculated for each type and summed together to provide the value designated by the summation sign (∑) in the preceding equation.

Plate Worksheet - Quantification of Bacterial Kinds and Numbers

Names_____ Lab Day_____

Culture Type_____

Number of colonies	Characteristics of Colony (as visible by unaided eye or w/ dissecting microscope: see below*)						Microscopic characteristics** (use 100X oil immersion objective)
	edge	sheen	silhouette	color	transparency	solidity	

Total number of colonies _____
Total number of kinds of bacteria colonies _____
Diversity calculation (H') _____

* Use the following terms within each category:
edge: smooth (i.e., perimeter not jagged) or rough (perimeter jagged)
sheen: shiny, semi-shiny or dull
silhouette: convex or flat
color: include color (e.g., light yellow, white) and any other color characteristics (e.g., pearl; red near perimeter, etc.)
transparency: opaque, translucent (light can pass through but not an image) or transparent (clear)
solidity: solid (maintains shape like gelatin, when plate is tilted) or semi-liquid (shape changes like a water balloon)

** Identify whether the organism is fungal or bacterial. If it is bacterial, identify whether it is gram-positive or gram-negative and bacillus (rod shaped) or coccus (spherical) in shape. If it is coccus, identify whether it is streptococcal (in a chain of more than two spheres) or staphylococcal (in grape-like clusters), or diplococcal (pairs of spheres).

Table 1.
Physical Features and Bacterial/Fungal Communities of Aged Plain and Chocolate Milk

Age/Type Milk	**Environmental Characteristics of Milk Culture (pH, Consistency, Odor, Color)**	**Organisms Present on TGY Plates (Bacteria: Gram +/–, Shapes; Yeasts or Fungi)**
Refrigerated plain		
24-hr plain		
4-day plain		
8-day plain		
Refrigerated chocolate		
24-hr chocolate		
4-day chocolate		
8-day chocolate		

Discussion

1. Describe the changing sequence of organisms and corresponding environmental changes during succession in plain milk. Do the results of your investigation match your hypothesis?

2. Describe the changing sequence of organisms and corresponding environmental changes during succession in chocolate milk. Do the results of your investigation match your hypothesis?

3. Compare succession in plain and chocolate milk. Propose reasons for differences.

4. Propose an experiment to test the environmental factors and/or organisms changing in your proposed scenario for milk succession.

Part 2 - Fungal Diversity & Evolution

In this part of the laboratory, you will have the opportunity to examine additional fungi and their cellular morphologies. The fungal stations are set up on the side-front bench. We have live material and also some prepared slides to help you locate the innovations that are important to the invasion of land by fungi. The next two pages have questions for you to answer about various structures and characters of certain groups.

One major task for this afternoon is to understand the life cycles of fungi and to get more familiar with the processes that occur in the various structures (two fungal life cycles charts from your text are on p. 14). Finally, be sure to step outside into the lobby to enjoy some of the benefits of bacterial and fungal growth and metabolism (after washing your hands, of course)! **Please hand in pp. 12-13 with the milk lab pages next week in lab (one set of pages per pair).**

Fungi

1. In what way were fungi essential to the invasion of land by plants? For one answer, examine the plant root cross section and describe the relationship that exists between plants and fungi in the mycorrhizae.

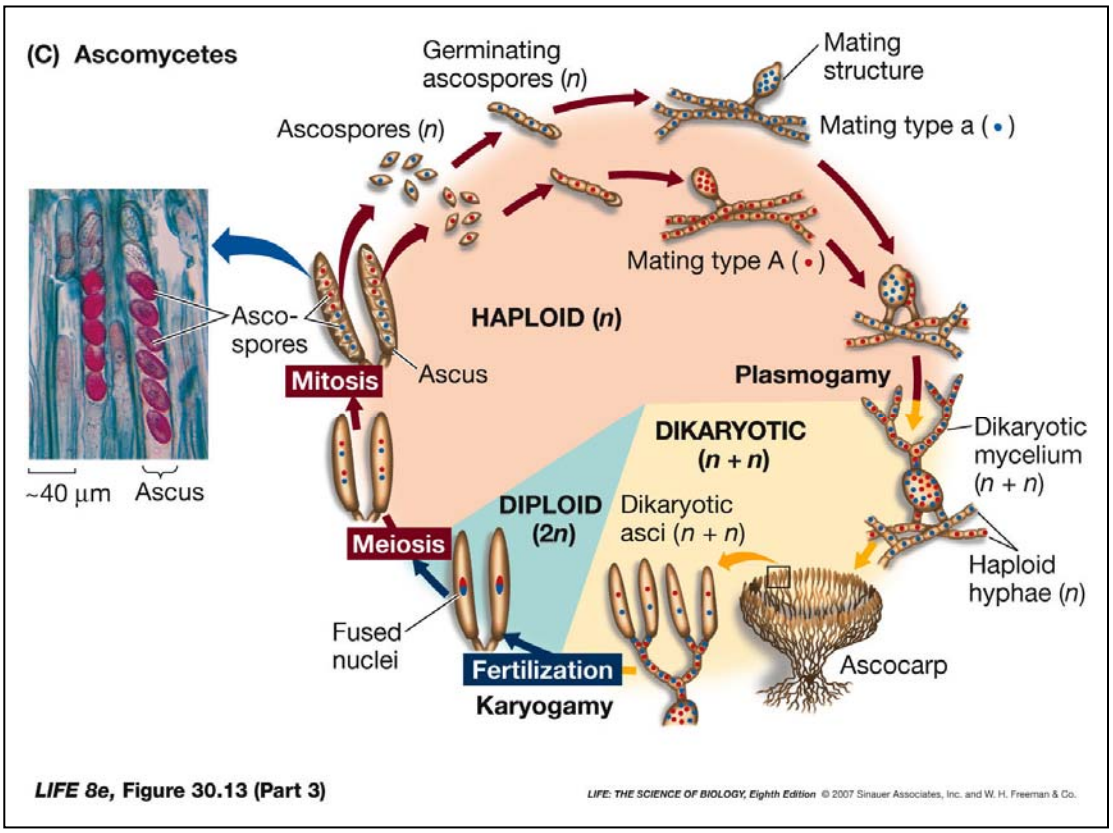
   What was this fungus providing the host plant, and what was it receiving in return?

2. Our most familiar fungi, the mushrooms, can be divided into two main groups depending upon how they release their gametes: the "ascomycetes" - sack fungi, and the "basidiomycetes" - club fungi (see the wall chart for features of each). Now examine the life cycle charts. What cellular processes distinguish the *dikaryotic* stage of these fungi from the *diploid* stage?

3. Examine the agar plate with colonies of unicellular yeast growing on it. To the visible eye, yeast colonies look very similar to bacterial colonies. What distinguishes yeast from bacteria at the cellular level?

4. Describe the symbiotic relationship that gives sourdough bread its distinctly tangy flavor.

5. The phylogenetic tree on p. 597 of your textbook indicates that fungi are more closely related to animals than they are to plants. What structural or physiological features make fungi different from plants and more similar to animals?


(C) Ascomycetes
Germinating ascospores (n)
Mating structure
Ascospores (n)
Mating type a (•)
Mating type A (•)
HAPLOID (n)
Asco-spores
Ascus
Mitosis
Plasmogamy
DIKARYOTIC (n + n)
Dikaryotic mycelium (n + n)
~40 µm
Ascus
DIPLOID (2n)
Dikaryotic asci (n + n)
Meiosis
Haploid hyphae (n)
Fused nuclei
Fertilization
Karyogamy
Ascocarp
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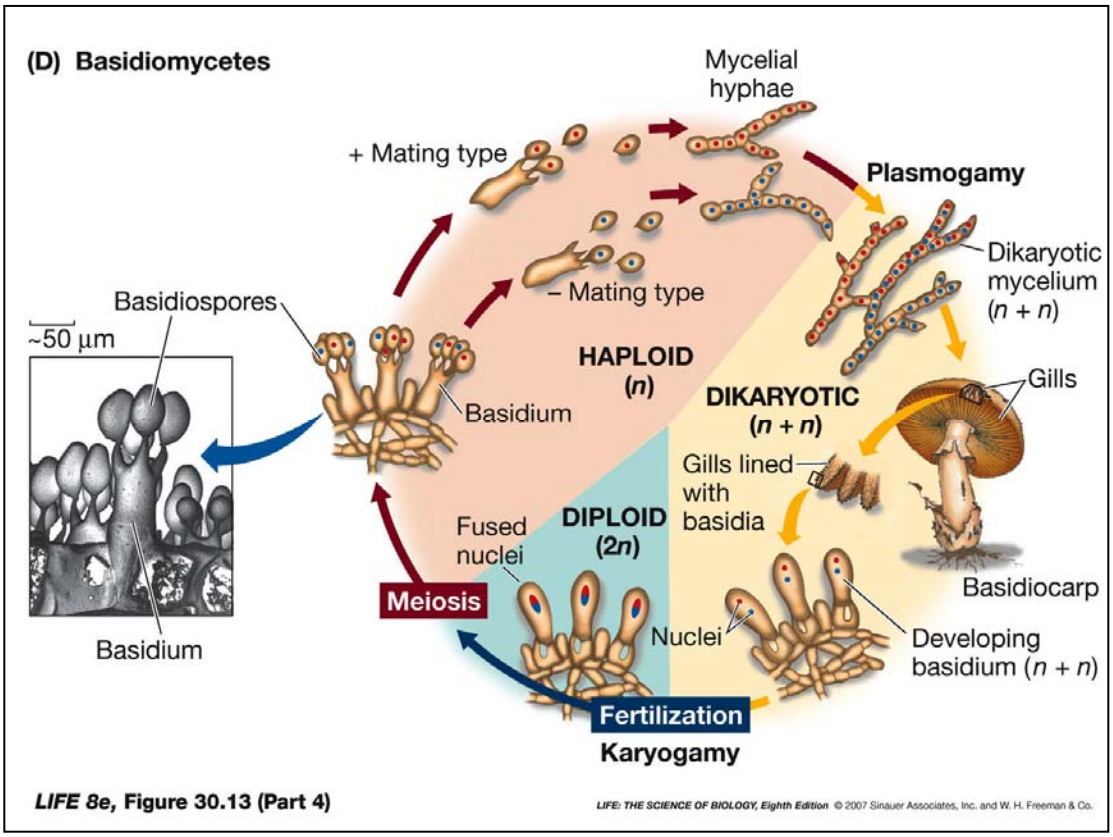
(D) Basidiomycetes
Mycelial hyphae
+ Mating type
Plasmogamy
Dikaryotic mycelium (n + n)
– Mating type
Basidiospores
~50 µm
HAPLOID (n)
Basidium
DIKARYOTIC (n + n)
Gills
Gills lined with basidia
Fused nuclei
DIPLOID (2n)
Meiosis
Basidiocarp
Nuclei
Developing basidium (n + n)
Basidium
Fertilization
Karyogamy
LIFE 8e, Figure 30.13 (Part 4)
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