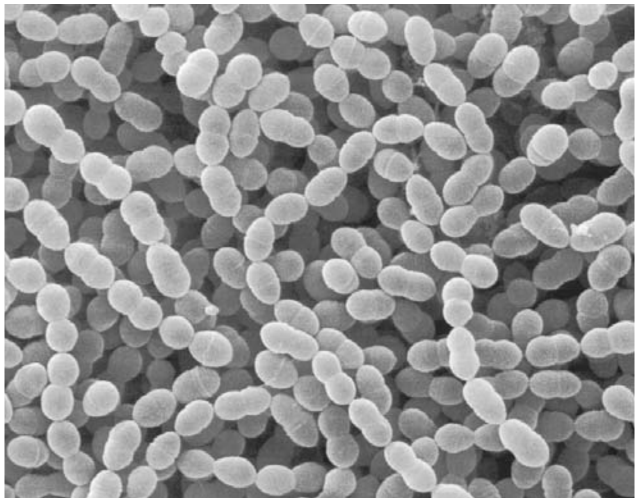

Biotechnology Explorer™

Microbes and Health Kit: "What Causes Yogurtness?"™

Catalog #166-5030EDU

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Store components of this kit at room temperature.

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Student Manual

Background

In the 1800's microbial diseases were a terrifying mystery. People sickened and died without apparent cause. It had long been suspected that contact with an infected individual was necessary for the transmission of disease, but this was not true for all diseases. Early microbiologists acted as detectives on the trail of a multitude of microbial killers. They were able to view bacteria from diseased individuals with microscopes, but how could they prove that the bacteria actually caused the disease? We will use Koch's postulates, a series of tests devised by Robert Koch, a German physician of the 1800s. Koch's postulates are widely used to prove that a particular microbe causes a particular disease.

Koch's postulates:

1. The microorganism must be found in all organisms suffering from the disease, but not in healthy organisms.
2. The microorganism must be isolated from a diseased organism and grown in pure culture.
3. The cultured microorganism should cause disease when introduced into a healthy organism.
4. The microorganism must be again isolated from the inoculated, diseased experimental host and identified as identical to the original specific causative agent.

Since it is dangerous and often unethical to experiment on humans, scientists often use model systems to simulate diseases in humans. Frequently, medical researchers will examine diseases in animals so that they can learn more about similar diseases in humans. You will use a model to test Koch's postulates. In this model, milk will represent a healthy individual. At times milk will develop a condition that causes it to thicken and turn into yogurt. This is the "yogurtness disease." You will play the role of a medical investigator from a time over a hundred years ago. You suspect that the yogurtness disease may be caused by something that is found in yogurt. You will use Koch's postulates to prove or disprove the hypothesis that microbes found in yogurt are the cause of yogurtness disease. Of course it is important to remember that real yogurt is a very healthy food and that any microbes found in yogurt are harmless and do not cause disease in healthy humans. Only a small minority of any bacteria cause disease in humans. In fact the "probiotic" (beneficial) bacteria found in yogurt may be helpful for digestion and may promote good health.

Ampicillin may cause allergic reactions or irritation to the eyes, respiratory system, and skin. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Wear suitable protective clothing. Ampicillin is a member of the penicillin family of antibiotics. Those with allergies to penicillin or any other member of the penicillin family of antibiotics should avoid contact with ampicillin.

Background for Instructors

Bacteria Are Everywhere

Bacteria are the single most successful form of life on the Earth. There are probably more bacteria, more species, and more total biomass of bacteria than any other lifeforms. Bacteria are found in soil, water, in and on animals, in and on plants, in and on humans, and even miles below the ground. There is speculation that bacteria or similar forms of life may exist on Mars or other planets.

Characteristics of Bacteria

Bacteria are one of the three great domains of life along with Eukarya (Animals, Plants, and Fungi) and Archaea (ancient bacteria-like organisms classified as a separate domain of life by Carl Woese in 1977). Bacteria and Archaea are classified as prokaryotes, single-celled creatures usually too small to be seen by the unaided eye. Bacteria are so small that it would take 5,000 to 50,000 in a row to stretch for an inch. Bacteria have no separate compartment (nucleus) to hold their DNA as eukaryotes do. Bacteria can sometimes, but not always, move by tiny tails called flagella. Bacteria sometimes grow connected to other bacteria forming chains. Some types of bacteria that may been seen in this lab grow connected in chains.

Bacteria as Pathogens

When we think of bacteria we usually think of disease. In fact, only a tiny minority of bacteria are capable of causing disease. Bacteria that do cause disease have played an enormous role in the history of humanity—cholera, typhus, the bubonic plague, tuberculosis, and other bacteria have sickened and killed millions. The development of antibiotics has greatly reduced the dangers of bacterial diseases. However, due to the overuse of antibiotics some bacterial strains (such as methicillin-resistant *Staphylococcus aureus* or MRSA) have developed antibiotic resistance leaving humanity exposed to the reemergence of old bacterial threats.

Bacteria can also spoil food such as milk. Milk is an ideal growth medium for bacteria and may contain both spoilage bacteria capable of souring milk, and pathogenic bacteria which might cause disease in humans, such as brucellosis, bovine tuberculosis, and scarlet fever. Milk is pasteurized by heating to 62.9°C for 30 min, or 71.6°C for 15 sec, and then cooling rapidly. Pasteurization destroys all pathogenic bacteria, and most but not all, spoilage bacteria. Thus milk still needs to be kept cool when stored. Grade A milk should contain less than 30,000 bacteria per milliliter.

History of Bacteriology

Anton van Leeuwenhoek of the Netherlands first saw bacteria through a microscope in 1676 and called them animalcules (tiny animals). Later Christian Gottfried Ehrenberg coined the term "bacterium" (meaning "small staff" in Greek) in 1828. In 1835 Agostino Bassi proposed the "germ theory of disease" which connected the spread of disease to unseen microorganisms, as prevously bacteria were thought to arise spontaneously in suitable environments. Louis Pasteur and John Tyndall showed that boiled broth grew bacteria only when exposed to the air thus disproving the theory of spontaneous generation. In 1875 Robert Koch was able to offer convincing proof of the germ theory by proving that anthrax was caused by bacteria. Koch's set of rules (Koch's postulates) for proving the cause of anthrax are the basis for assigning the cause of disease to a particular microbe. The postulates are also the basis for the experiments in this lab.

Types of Bacteria and Bacterial Colonies

There are several distinct morphologies or shapes of bacteria. The three major shapes are coccus (spherical), bacillus (rod-shaped), and spirillum (spiral). Cocci and bacilli can exist singly, in pairs (diplococci or diplobacilli), attached in long strings (streptococci or streptobacilli), or connected in other arrangements (staphylococci or staphylobacilli). There are various forms of spiral bacteria too, such as comma-shaped (*Bdellovibrio*), helical (*Helicobacter pylori*), or long twisted spirochete forms. It is best to examine fresh cultures as older bacteria are occasionally oddly shaped and may have lost motility.

Bacteria increase in number by binary fission (splitting in half). Some bacteria can divide every 15–20 minutes! A single bacterium on a solid medium, such as an agar plate, increases logarithmically so that overnight a single bacterium becomes millions or billions. These millions or billions of bacteria form a visible "colony" on an agar plate. A colony of bacteria can itself have a distinct form and be large or small. Some bacterial colonies are so small that they cannot be seen with the unaided eye. Colonies may be circular, irregular, or branching. The edge of the colony may be smooth, wavy or serrated. The colony may be flat, raised or raised only in the center.

Bacteria are also differentiated by their cell walls. Some have thick cell walls made of peptidoglycan molecules. The cell walls of these bacteria take up a dye called Gram stain and thus are called Gram-positive bacteria. Other bacteria have thinner cell walls that do not absorb Gram stain and thus are called Gram-negative bacteria. The lactic acid bacteria found in yogurt are Gram-positive bacteria. The HB101 K-12 *E. coli* bacteria provided in this kit are Gram-negative bacteria.

Bacterial Metabolism

Like all living things bacteria require food, often in the form of sugars, to gain energy. Bacteria break down sugars chemically into other molecules using enzymes. Enzymes are large proteins that speed up chemical reactions. This process of bacterial metabolism is often called fermentation.

Some bacteria require oxygen from the air to grow and are called aerobes. Other bacteria grow only in the absence of oxygen and are called anaerobes. Some bacteria can grow either with or without oxygen and are referred to as facultative anaerobes. Aerobic bacteria use oxygen to break sugar into intermediate products and then finally into carbon dioxide and water. Lacking oxygen, anaerobic or facultative anaerobic bacteria usually do not reduce sugars completely to carbon dioxide and water. Often these bacteria convert sugar into pyruvic acid and then convert the pyruvic acid into other by-products.

Yogurt forming bacteria are anaerobes and break down milk sugar (lactose) into pyruvic acid and then into lactic acid using enzymes. Lactic acid is the by-product or waste product made by lactic acid bacteria. Lactic acid also lowers the pH of milk making it acidic. The acidic conditions cause casein (a common protein in milk) to denature (or curdle) and become more solid. In addition the acidic conditions inhibit the growth of other microorganisms that might spoil the yogurt. Thus lactic acid causes the yogurt to stay fresh, while at the same time remaining digestible by people who can break lactic acid down for additional energy. Other bacteria can break down sugars and pyruvic acid and make other by-products. The *E. coli* bacteria break sugar down into succinic acid, ethanol, acetic acid, formic acid, and lactic acid.

Koch's Postulates

By the mid-19th century, the famous French scientist Louis Pasteur had conducted extensive studies on the role of bacteria in fermentation, and had shown conclusively that germs did not spontaneously appear in susceptible hosts (spontaneous generation), but rather needed to come in contact with the host first. There was already a prevailing assumption at the time that microbes were in some way connected with disease, but whether their presence was the cause or just a result of disease was unclear. Furthermore, many infected tissues contained more than one type of microorganism. This made it difficult to define with certainty the role any particular type of bacterium played in disease. The work of Pasteur and others, along with improved techniques in microscopy and the discovery of semi-solid culture media, all paved the way for the work of Robert Koch.

Koch had been studying anthrax, a deadly disease that infects both humans and animals, and he noticed that certain rod-shaped bacteria and their spores were characteristically found in the tissues of sick sheep. He meticulously isolated these bacteria, which he named *Bacillus anthracis*, and grew pure cultures in a medium consisting of the aqueous humor of cattle or rabbit eyeballs. Next, he introduced the bacteria from the cultures into healthy rabbits. When the rabbits subsequently developed symptoms of anthrax, Koch again isolated the bacteria from the rabbit tissue and observed them under the microscope to confirm that they were indeed the same ones he had seen in his original culture.

The steps he used are now known as "Koch's postulates." Meeting the criteria laid down by Koch is referred to as "satisfying Koch's postulates" and is considered the standard evidence required to show that a microorganism causes a particular disease.

To demonstrate Koch's postulates, students must do the following:

- Describe and record the symptoms shown
- Isolate and identify the suspected pathogen from the infected material and establish a pure culture
- Use the pure culture to infect new material. Describe and record the symptoms shown by the material. Check that these are the same as their original observations
- Again isolate and identify the organism

Beneficial Bacteria and Yogurt

Despite our longstanding association of bacteria with disease, most bacteria are essentially harmless. In fact, many bacteria are beneficial. Bacteria break down waste organic material. *Rhizobium* bacteria take nitrogen from the air and convert it into a usable form (fixation). Intestinal bacteria break down indigestible material and synthesize nutrients. Some types of bacteria are necessary for the manufacture of certain food products, such as cheese, kimchi, sour cream, pickles, and yogurt.

Yogurt is made by adding specific strains of bacteria into milk, which is then fermented under controlled temperatures and environmental conditions. The bacteria ingest natural milk sugars and release lactic acid as a waste product thus making the milk acidic. The increased acidity causes casein (the most common milk protein) to tangle into a solid mass (called curd) in a process called denaturation. The increased acidity (the usual pH of yogurt is 4–5) also inhibits the growth of other dangerous bacteria. To be classified and sold as yogurt in the United States it is required that yogurt must contain the bacteria strains *Streptococcus thermophilus* (*Streptococcus salivarius subsp. thermophilus)* and *Lactobacillus bulgaricus* (*Lactobacillus delbruecki subsp. bulgaricus*). Often these two are cocultured with other lactic acid bacteria for taste or health effects including *Lactobacillus acidophilus*, *Lactobacillus casei,* or *Bifidobacterium* *bifidum*. In most countries, a product may be called yogurt only if live bacteria are present in the final product. A small amount of live yogurt can be used to inoculate a new batch of yogurt, as the bacteria reproduce and multiply during fermentation. Pasteurized products, which have no living bacteria, are called fermented milk. In the United States yogurt must contain at least a billion viable bacteria per gram at the time of manufacture and at least a million viable bacteria per gram at the expiration date.

Yogurt has nutritional benefits beyond those of milk—people who are lactose intolerant often enjoy yogurt without ill effects, apparently because live yogurt cultures contain enzymes which help break down lactose inside the intestine. Yogurt also has medical uses, in particular for a variety of gastrointestinal conditions, such as preventing antibiotic-associated diarrhea.

In this lab students will isolate the bacterial strains found in a yogurt sample on agar in a petri dish, then use those same strains to inoculate fresh milk to find out if they can reproduce the same yogurt. Students should be able to conclude that the acidic, solidified nature of yogurt is caused by bacteria acting upon milk.

Antibiotics

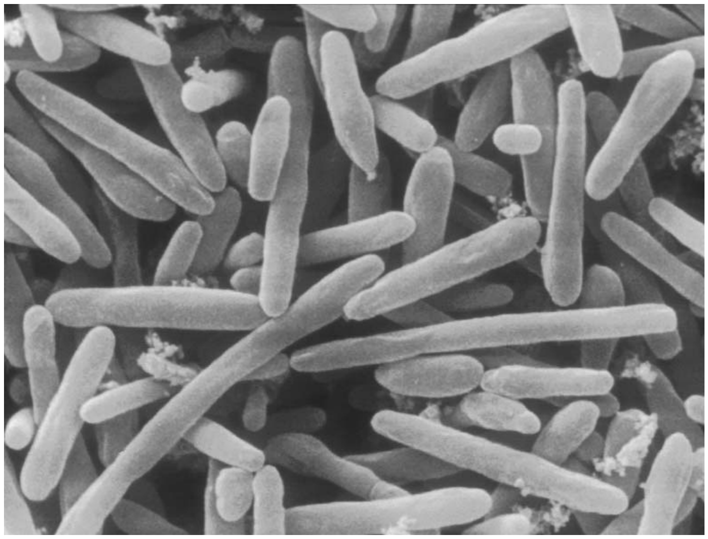
Early attempts to treat bacterial infections sometimes employed substances, such as arsenic or strychnine, that were nearly as toxic to humans as to bacteria. In 1928 Alexander Fleming discovered penicillin, a compound produced by mold, that inhibited the growth of bacteria without serious harmful effects upon humans. Many different types of antibacterial antibiotics have been discovered since that time. These antibiotics have vastly reduced the incidence of bacterial disease. Modern society has almost forgotten how great the dangers of bacterial disease once were. Careless misuse of antibiotics now threatens a return of potent bacterial diseases. Massive amounts of antibiotics are used in animal feed inadvertently selecting for the growth of bacteria resistant to many classes of antibiotics. People often needlessly take antibiotics for viral infections – again selecting for the growth of antibiotic resistant bacteria. In addition patients often discontinue use of antibiotics as soon as they feel better leaving the most resistant bacteria in place to start a new round of infection.

Antibacterial antibiotics are either bactericidal (kill bacteria) or bacteriostatic (prevent bacteria from dividing). There are many different modes of action for antibiotics. For instance, some inhibit the function of important enzymes present only in bacteria and not in mammals. Others destroy components of bacterial cell walls that are not used in mammalian cells.

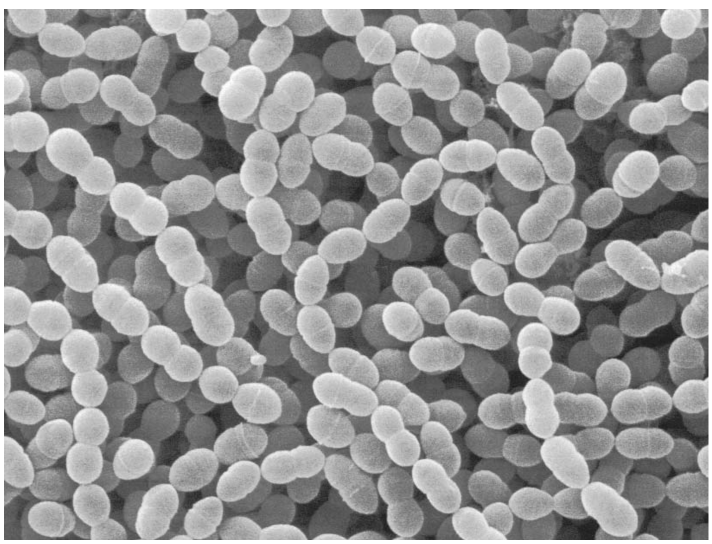
The antibiotic ampicillin is included in this kit both as an additional control and as a tool to allow further experimentation. Ampicillin is a beta-lactam antibiotic similar to penicillin and amoxicillin. It inhibits Gram-positive bacteria and some Gram-negative bacteria, such as *E. coli,* and it acts by preventing the synthesis of bacterial cell walls eventually leading to the death of the bacteria. Ampicillin is widely used in molecular biology as a selective agent since the gene for resistance to ampicillin (encoding for the beta-lactamase enzyme) can be inserted into bacteria on plasmids that may also carry genes of interest to scientists. Those bacteria that survive on ampicillin containing media will also have the gene of interest.

Sterile Technique

When culturing bacteria it is important to avoid contamination. Contaminating bacteria and molds are found everywhere, including on hands and lab benchtops, so it is important to avoid these surfaces. The round circle at the end of inoculating loops and the surfaces of agar plates should not be touched or placed onto potential contaminating surfaces. Wipe down lab benches with 70% alcohol or a 10% bleach solution wearing appropriate safety equipment.



Lactobacillus bulgaricus
(rod-shaped)



Streptococcus thermophilus
(spherical-shaped)

Quick Guide

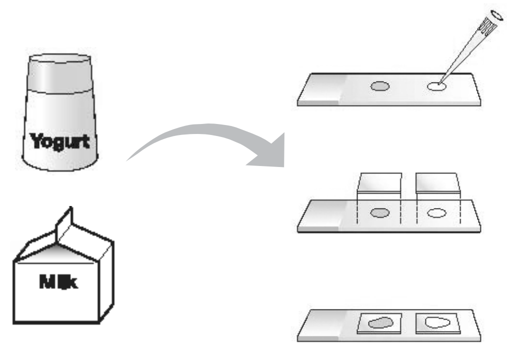
Lesson 1

Postulate 1: Identify possible pathogens

1. Compare yogurt and milk with respect to appearance, smell, and pH. Record observations.

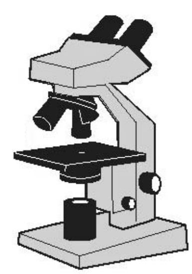
2. Label left hand edge of slide "yogurt" and right hand edge “milk”.

3. Dip toothpick in yogurt, mix with a drop of water on left hand side of slide, and cover with cover slip.



4. Add drop of milk to right hand side of slide and cover with cover slip.

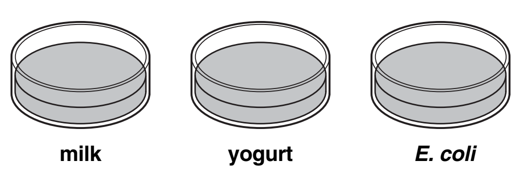
5. Observe yogurt and milk under the microscope. Describe and draw what you see.



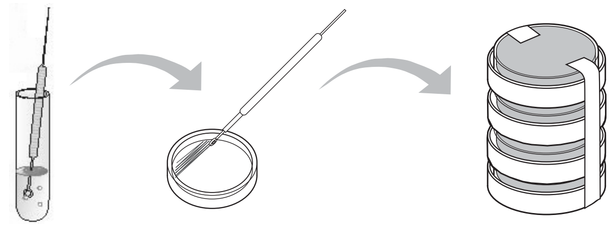
6. Repeat steps 1–5 with a different brand of yogurt.

Postulate 2: Isolate and culture suspected pathogens

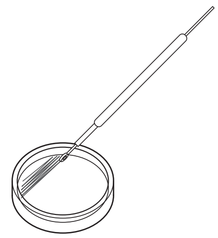
7. Label 3 LB sugar agar plates on the bottom (not the lid) with your initials and one as "milk", one as "yogurt", and the third as "*E. coli*".



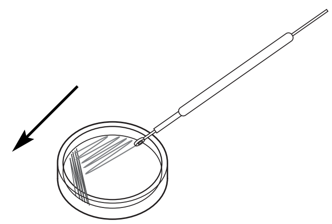
8. Streak milk onto milk plate for single colonies. Streak yogurt onto yogurt plate for single colonies as above. Streak *E. coli* onto *E. coli* plate for single colonies as above.



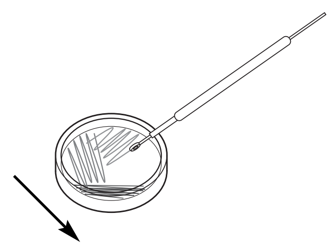
   A) Streak for single colonies by gently rubbing the loop back and forth in the top left corner of the plate about 10 times. Stay in the top left quadrant of the plate and do not break the surface of the agar.



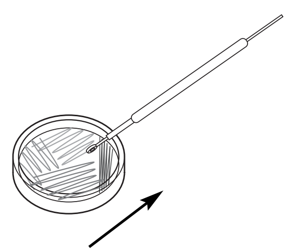
   B) Rotate the plate 45° and using the same loop draw the loop through one end of the first streak. **Do not dip the loop back into the starting material**. Then rub the loop back and forth in the second quadrant about 10 times. Avoid passing the loop into the first streak.



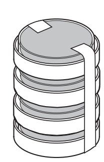
   C) Rotate the plate 45° and using the same loop draw the loop through one end of the second streak and rub the loop back and forth in the third quadrant about 10 times. Avoid passing the loop into the first and second streaks.



   D) Rotate the plate 45° and using the same loop draw the loop through one end of the third streak and rub the loop back and forth in the fourth quadrant about 10 times avoiding all previous streaks.



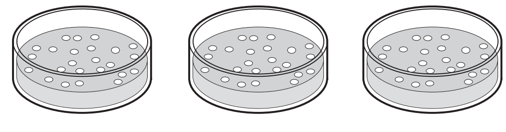
9. Invert the plates and place in incubator at 37°C for 24–48 h.



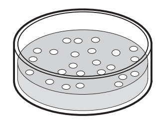
Lesson 2

Postulate 2 continued: Isolate and culture suspected pathogens

1. Obtain plates from previous lesson. Count the individual colonies on each plate. Record results.



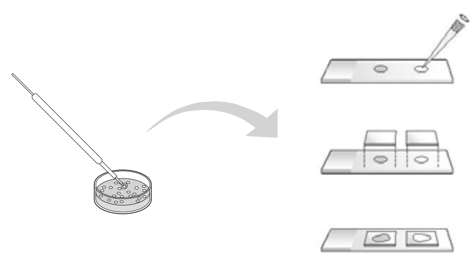
2. Observe colonies. Use a magnifying glass if available. Record how many different types of colonies you have on each plate. Use a marker to circle one of each type of colony and label with a number on the bottom of the plate.



3. Describe the appearance of each numbered colony.

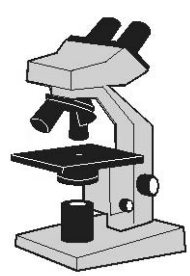
4. Label some slides according to your colony numbers. Use one slide for two samples as in the first lesson.

5. Pick a numbered colony from the yogurt plate, mix with a drop of water on right hand side of the appropriately numbered slide, and cover with a cover slip.



6. Repeat with the other numbered colonies from the yogurt, milk, and *E. coli* plates.

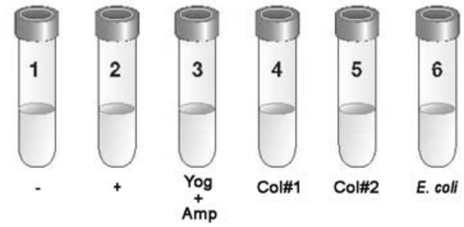
7. Observe colonies under the microscope. Describe and draw what you see.



8. Compare the bacteria with your descriptions of those observed in the yogurt in the first lesson.

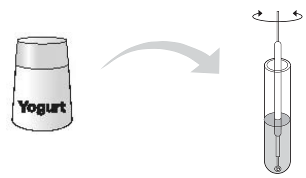
Postulate 3: Inoculate healthy individual with pure culture of suspected pathogen

9. Label 6 tubes of milk as follows:
   Tube 1 Negative control
   Tube 2 Yogurt (positive control)
   Tube 3 Yogurt + amp
   Tube 4 Yogurt Colony #1
   Tube 5 Yogurt Colony #2
   Tube 6 *E. coli*

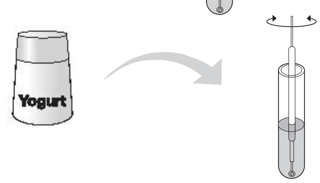


10. Add 10 µl or 1 drop of ampicillin to tube "Yogurt + amp".

11. Dip a fresh inoculation loop into the yogurt and swirl the loop into tube "positive control".

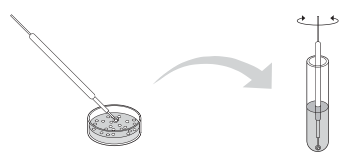


12. Use the same loop to dip into the yogurt again and swirl into the "Yogurt + amp" tube.

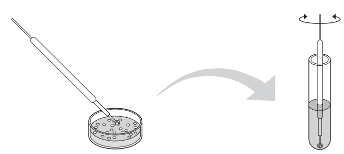


13. Identify two colonies on the yogurt agar plate that you investigated in the previous lesson of different types, if possible. Number the colonies 1 and 2 on the bottom of the plate and record which is which. If there is only one type of colony on your yogurt plate then number two similar colonies.

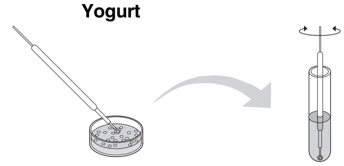
14. Using a fresh inoculation loop, pick colony #1 and transfer it to the tube "yogurt colony #1".



15. Using a fresh inoculation loop, pick colony #2 and transfer it to the tube "yogurt colony #2".



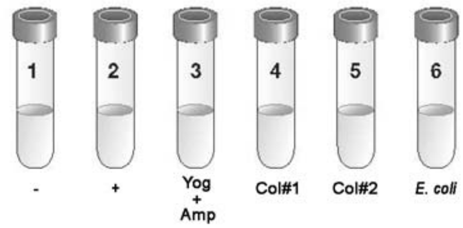
16. Using a fresh inoculation loop, pick an *E. coli* colony and transfer it to the tube "*E. coli*".



17. Place the tubes in an incubator or water bath at 37°C for 24–48 h.

Lesson 3

Postulate 4: Isolate and identify suspected pathogen from newly diseased individual

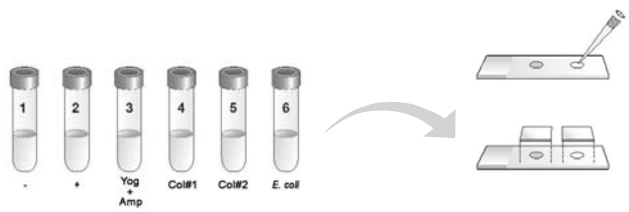


1. Obtain milk tubes and yogurt agar plate from previous lesson. Describe each milk culture with respect to appearance, smell, and pH.

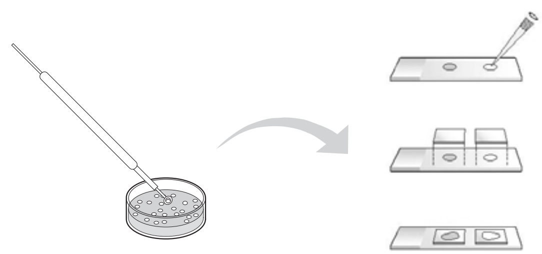
2. Label 3 slides according your milk tube labels. Use one slide for two samples on the right and the left as in the first lesson.

3. Label a fourth slide yogurt colony #1 on the right and yogurt colony #2 on the left.

4. Prepare slide samples of each milk culture for viewing under microscope as in previous lessons. For solid cultures, dip toothpick in culture and mix with a drop of water. For liquid cultures, add a drop to the slide. Cover with cover slip.



5. Pick a colony from the yogurt plate similar to that used to start the yogurt cultures in tube 4 (i.e. the same colony type as yogurt colony #1). Mix colony with a drop of water on right hand side of the appropriately numbered slide and cover with cover slip. Repeat with yogurt colony #2 on the left of the slide.



6. Observe slides under the microscope. Describe and draw what you see.

7. Using the microscope compare any bacteria in the newly infected cultures in milk tubes 4 and 5 with the pure bacteria used to inoculate these cultures. Are they the same?