

Yogurt Making – How Does Carbohydrate Type Affect Yogurt Chemistry?

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This is an inquiry-based lab intended to be used in a majors- or non-majors introductory biology course. There are several published and commercial examples of laboratory activities for biology courses that involve making yogurt or culturing the bacteria isolated from yogurt; however, these labs are typically completely directed or demonstration labs that do not allow for hypothesis generation or experimentation by the students. The main goal of this lab is to allow students to generate hypotheses about what chemical changes will occur to various substrates (different types of milk) during the process of lactate fermentation with *Lactobacillus* and related microbes. Students are provided with a basic yogurt making protocol and use test strips to measure pH and glucose concentration. Students devise their own methods for measuring yogurt consistency. This is a two-session lab exercise that requires students (or lab staff) to remove yogurt from incubators after 18-24 hours. The materials are inexpensive, and this lab can be scaled to large enrollment laboratory courses.

Keywords: Yogurt, fermentation, lactate, lactic acid, *Lactobacillus*

Introduction

The biochemical steps and overall process involved in fermentation are often challenging concepts for introductory biology students. This two-session lab presents an easy, inexpensive, directed inquiry exercise that allows students in introductory majors- or non-majors biology to explore the biochemical changes that occur to substrates during the process of lactate fermentation. Yogurt making provides an accessible, interesting platform on which to investigate one aspect of metabolism. The objectives of this lab are to increase understanding of the chemical changes (changes in pH, carbohydrate structure and concentration, and consistency) that occur to milk during the process of lactate fermentation with *Lactobacillus* and related microbes, and to engage students in hypothesis generation, experimental design, team work and data analysis.

Prior to beginning this lab students should have a basic understanding of the process of lactate fermentation and a familiarity with organisms capable of this process. This background can be provided in lecture or in self-guided study / pre-lab reading. Upon arrival in lab, students are asked to make predictions about how a few basic measurements (pH, glucose concentration, consistency) will differ between milk and yogurt. Students investigate these measures in various different starting substrates that can be used to make yogurt – cow's milk, lactose reduced cow's milk, soy milk, almond milk, etc. Students are then provided with a simple proto-

col for making basic yogurt using dry yogurt starter (previously prepared yogurt can be used as a starter if desired). Each student group makes cow's milk yogurt plus one or two additional yogurts using non-traditional substrates. It is suggested that, across an entire lab section, the substrates available be distributed so that all possible substrates have been tested by two groups. Students place their warmed milk plus yogurt starter in laboratory incubators at ~37°C, remove set yogurt 18-24 hours later and either examine the yogurt or refrigerate it until the next lab session. The next laboratory period (yogurt can be stored up to two weeks at 4°C) students test yogurt for pH and glucose concentration and for consistency and compare to initial measurements. Each student group then analyzes their data and shares results with the rest of the class.

Student Outline

Objectives

1. Investigate the chemical processes involved in lactate fermentation by making yogurt.
2. Design an experiment to examine how different carbohydrates present in different commercially available “milk” products affect the fermentation process in yogurt.
3. Explain the following terms:
 - Fermentation
 - Glycolysis
 - Oxidized / reduced coenzymes
 - ATP
 - NADH / NAD⁺
 - Pyruvate
 - Lactate
 - Ethanol
 - Lactate fermentation
 - Alcoholic fermentation
 - Lactose
 - Sucrose

Background

We have already discussed **aerobic respiration**– the breakdown of glucose in the presence of oxygen to produce large numbers of ATP molecules per molecule of glucose. Today we will investigate how organisms break down glucose without consuming oxygen – **glycolysis** followed by **fermentation**. Glycolysis is the common pathway for the beginning steps of both aerobic respiration and fermentation. After glycolysis, a molecule of glucose has been converted into two molecules of 3-carbon **pyruvate**, and a net total of two molecules of ATP and two molecules of the **reduced coenzyme** NADH have been produced. In the absence of oxygen, NADH is **not** transported into mitochondria to be further broken down in the citric acid cycle and electron transport phosphorylation. Because the cell needs to continue the process of glycolysis to produce more ATP for cellular functions, it needs to recycle its NADH back to NAD⁺. In the process of **lactate fermentation**, pyruvate is reduced to another 3-carbon molecule called **lactate** by the enzyme **lactate dehydrogenase** (Freeman, 2011). The H⁺ and accompanying electron from NADH are received by pyruvate and pyruvate is converted to lactate as seen in Fig. 1.

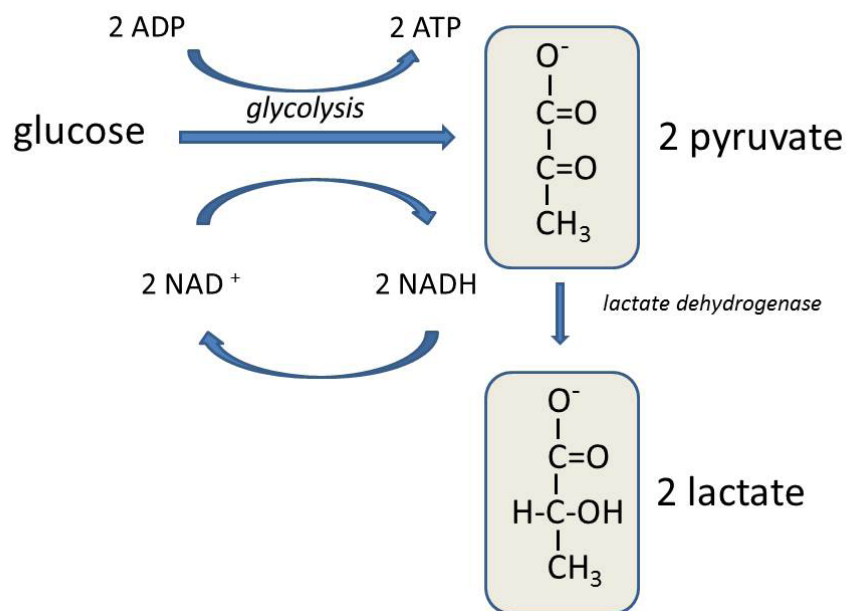


Figure 1. Lactate fermentation. Note the **substrates** and **products** of this overall reaction.

This type of fermentation (called **lactate** or **lactic acid fermentation**) occurs in animal muscle cells in anaerobic conditions and in some kinds of bacteria, like *Lactobacillus* and related bacteria –which are commonly used to make yogurt, one kind of fermented milk. Yogurt is simply the result of these lactate fermenting bacteria consuming **lactose** (the disaccharide present in milk) to form the two monosaccharides glucose and galactose (Fig. 2).

These monosaccharides are converted to pyruvate during glycolysis and ultimately to lactate (lactic acid). The production of this acidic molecule changes the structure of milk protein and causes the thickening typical of yogurt, and it is also responsible for the tangy (acidic) taste of plain yogurt. The microbes involved in commercial yogurt production (*Lactobacillus*, *Bifidus*, *Streptococcus thermophilus* and related organisms) are facultative anaerobes – which means that fermentation will progress in the presence of some oxygen, although several species of microbes are more efficient at fermentation in the absence of oxygen (Horiuchi *et al.*, 2009). Although lactose is the preferred starting substrate for these microbes, many of these microbes are able to digest other simple and complex carbohydrates to some extent (Rathore *et al.*, 2012).

Lactate fermentation is not the only fermentation pathway – yeast perform a process called **alcohol fermentation**, in which the final product is ethanol (a 2-carbon molecule) and a molecule of carbon dioxide (Freeman, 2011). Even though the end products are different (ethanol and CO₂), alcoholic fermentation recycles NADH back to NAD⁺, just as in lactate fermentation. People utilize the products of alcoholic fermentation to produce wine (fermented grape carbohydrates where ethanol is the desired product), bread (fermented wheat carbohydrates, where the rising of bread through the production of gaseous CO₂ is the desired product – the ethanol is baked out), and beer (where both the ethanol and the bubbles of CO₂ are desired).

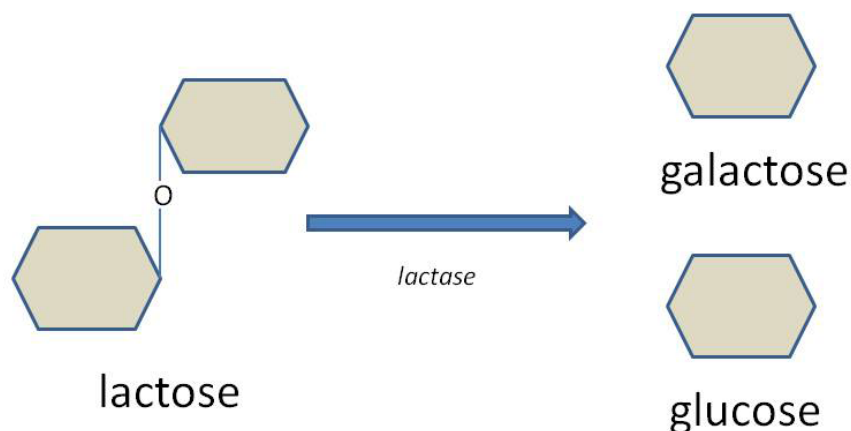


Figure 2. Hydrolysis of the disaccharide lactose into the monosaccharides glucose and galactose by the enzyme lactase (β -galactosidase).

Week 1 Protocol

Safety Note: although we are using food grade products for this lab, **NEVER taste anything in lab.** The lab glassware, while clean, is used for many other lab activities and there is always a chance of contamination. I will provide an opportunity outside the lab room for you to taste plain and soy milk yogurt

Objectives: Today, you will investigate how various carbohydrates are fermented by yogurt bacteria. You may have seen “soy yogurt” and “almond yogurt” in stores. These products are produced by lactate fermentation, just like normal dairy yogurt; however, they do not contain lactose, but instead contain other simple carbohydrates that can act as substrates for fermentation.

Question you will address: how do different starting carbohydrates affect lactate fermentation and yogurt production?

We have several different kinds of “milk” for you to experiment with:

- Normal cow’s milk (2% milk fat),
- Lactaid milk (2% milk fat),(Lactaid is cow’s milk treated with the enzyme lactase to break down the disaccharide lactose into its constituent monosaccharides),
- Several plant based products:
 - unsweetened soy milk
 - unsweetened almond milk
 - unsweetened coconut milk

Your specific task is to compare how the following variables change during the process of yogurt making – from the beginning substrate (unheated dairy milk/alternate milk product) to the end product (fermented milk/alternate milk product –yogurt):

- pH
- monosaccharide content
- consistency

Step 1: Each group will compare the process of fermentation in normal 2% cow's milk to one other “milk” product of their choosing (see above list). Coordinate with the other group at your table and make sure each group chooses a different alternate milk product. Record your choice of alternate “milk” here _____.

Step 2: *Before* you begin your experiment answer the following questions and make some predictions about what you expect for the following:

2% Dairy (cow) Milk

- What types of carbohydrates do you think are in milk?
- What will the pH be for milk? _____
- What will the monosaccharide content be? (high, medium, low) _____
- What will the consistency be? (runny, thick) _____

Alternate “Milk” (record your alternate “milk” type here)[_____]

- What types of sugars do you think are in this substance? (Examine ingredients list)
- What will the pH be? _____
- What will the monosaccharide content be? (high, medium, low) _____
- What will the consistency be? (runny, thick) _____

Step 3: Measure your starting substrates:

- Measure consistency: Work with your group to devise a repeatable, quantitative method to determine consistency of both milk and set yogurt. Various supplies are provided on the side bench – including glassware, cheesecloth, glass stir rods, stopwatches and funnels. There is also a container of commercial yogurt. Be creative in devising a consistency measure, but it must be repeatable. Test your method. Once you are satisfied with your consistency method, explain your protocol to your instructor. Carefully record the steps of your consistency method in your lab handout so that another student could repeat the procedure.
- Measure glucose concentration and pH. Use the test strips to test the pH and monosaccharide content of your substrates. Pour a small amount of substrate into a clean container and dip the test strip into the substrate. Find test spots for pH and glucose. Compare test strip to color scale on bottle and record data on the next page.

Step 4: Record your measurements -construct a data table in your lab notebook to record your pH, glucose content and consistency data from the beginning of the experiment. Make sure to give your table a title.

Step 5: What sort of control do you need? Discuss controls with your lab group and have your controls approved by your instructor.

Step 6: Make yogurt! Below is a generic protocol for making yogurt. So that we can compare class results, I would like everyone to follow the same protocol. Each group needs to prepare two 50 ml samples of yogurt for each starting milk product.

Basic Yogurt Protocol:

- a) Heat 110 ml of starting milk to 85°C in a 250 ml beaker on hot plate.
- b) Stir milk occasionally or use magnetic stir bar
- c) Once the milk reaches temperature remove IMMEDIATELY from hot plate with a hot glove. CAREFULLY!
- d) Do not let milk boil
- e) Place beaker in cool water / ice bath
- f) Stir milk occasionally while cooling
- g) When milk reaches 45°C you are ready to move to the next step.
- h) Do not let milk temperature drop below 35°C
- i) Add 1 g of yogurt starter per 100 ml of cooled milk
- j) Stir to dissolve yogurt starter
- k) Aliquot milk into 50 ml beakers
- l) Cover beakers with plastic wrap or aluminum foil (not airtight)
- m) Use lab tape to label all beakers with your lab group name and contents of beakers
- n) Place beakers into incubator at 37°C

Step 7: Share data on starting substrates and consistency methods

- a) Record your lab group's milk type and write your values for pH and monosaccharide content on the chart on the board.
- b) Each group will explain how they plan to measure consistency of the yogurt.

Step 8: Discuss these questions with your group, and then we will discuss as a class.

- a) Why do the monosaccharide values for the various milks vary?
- b) Is there much variation in pH?
- c) What milk types have the largest difference between glucose concentrations?

Step 9: Preparation for next week: Discuss with your lab group and write down your predictions

- a) Do you think the various substrates will all "set" into yogurt? Why or why not?
- b) What do you think will happen to the pH values of the yogurt compared to the milk? Why?
- c) How do you think the monosaccharide levels of the yogurt will compare to the starting substrate? Why?

Step 10: TOMORROW: 18-24 hrs later. One of your lab members needs to come to lab to remove the yogurt from the incubator and put it in the refrigerator. Place the beakers of yogurt on the shelf labeled with your lab section.

Week 2 Protocol

Objectives: You will collect data on the samples of yogurt that you prepared last week.

Step 1: Remove yogurt containers from refrigerator and measure the following for both yogurt samples. Record data in the data table you constructed last week.

- a) pH
- b) monosaccharide content
- c) consistency

Step 2: Share results with class

- a) Record your lab group's milk type and write your values for pH and monosaccharide content on the chart on the board.
- b) Write thicker / thinner / no change for consistency after yogurt production for each sample

Step 3: Discuss the following questions with your lab group, and then we will discuss as a class

- a) Has the monosaccharide content of the cow's milk changed, if so, how and why?
- b) What has happened to the monosaccharide content of the Lactaid?
- c) Why has the pH decreased? How does this relate to the physiological process of lactate fermentation?
- d) Why does the consistency of yogurt vary for the different starting substrates?

Writing Assignment:

This experiment will be the basis for your first lab report. [Specific instructions, due dates, etc. for writing lab reports provided to students separately.]

Materials

I provide the following materials in a centralized location so that students make conscious decisions about what materials are needed and when they need them.

- Milk and alternate milk products. 200 ml of cow's milk for each lab group, plus 200 ml of alternate milk product per lab group (all 6 lab groups use cow's milk, plus one additional milk product so that the alternate milk types have at least two replicates in the lab as a whole)
 - Low fat (2%) cow's fat milk
 - 2% fat Lactaid milk (milk treated with the enzyme lactase)
 - Soy "milk" (unsweetened)
 - Almond "milk" (unsweetened)
 - Coconut milk (unsweetened - canned. ** There is a product called coconut water which should NOT be used – it is too thin and is often sweetened.)
- Large (32 oz or ~946.35 ml) container of plain unsweetened yogurt or lab-made yogurt for testing consistency (on first lab day). One container sufficient for 15-20 lab groups.
- Urinalysis test strips (with glucose and pH indicators) 6-8 strips per lab group. (Suggested supplier: Glucose test strips, Diascreen 3. Fisher catalog # 02-675-279 (\$42 for 42 strips))
- Yogurt starter – 2 g per group (several brands available in grocery store – usually in cold section next to the packaged yogurt: Yogourmet dry starter – one box = 30g of starter for about \$6, sufficient for 15 lab groups doing two milk products apiece, or EuroCuisine dry starter one box = 50 g of starter for about \$15). Yogurt starter needs to be refrigerated until use. Starter can also be ordered from internet sellers like Amazon.
- ALTERNATE to starter: if dry yogurt starter is unavailable or too expensive, purchase unsweetened plain yogurt (NOT Greek yogurt) to use as a starter. The recommendation is 1 TBS (~10-15 g) of prepared yogurt per 100 ml of milk added once the milk has cooled to 45 °C)
- Glassware (large and small beakers) (2, 250 ml or larger beakers, and 6, 50 or 100 ml beakers per group)
- Hotplate / stir plate (one per group)
- Laboratory incubator at 37°C (this is an incubator-space intensive project compared to petri dishes, so multiple incubators might be necessary if multiple lab sections running concurrently)
- Thermometers (one per group)
- Weigh boats (one per group)
- Laboratory balance (one per class)
- Magnetic stir bars if using stirring hotplates
- Ice / cool water baths: plastic basins, large Tupperware or other containers for ice baths
- Materials for measuring consistency of yogurt (materials will vary depending on methods devised by lab groups – I usually let them use whatever general materials we have available in the lab)

- Funnels
- Cheese cloth (4-6 sheets per group, if needed)
- Spoons (an assortment of plastic, metal and wooden spoons or spatulas for measuring consistency)
- Disposable plastic 1.5 ml pipettes

Notes for the Instructor

At the beginning of the laboratory period, the instructor should guide the students to the question that is being asked. Ask students how many have tasted plain yogurt, soy yogurt, or almond yogurt. Ask them how they think yogurt is made. Ask students why they think plain yogurt tastes tangy, why yogurt has a different consistency than milk. The instructor should help students make tangible connections between the metabolic process of lactate fermentation and the chemical and physical changes that students will observe during the process of making yogurt.

It is crucial for all groups to follow the same protocol for making the yogurt so that comparisons can be at the class level. If at all possible, purchase the same percent fat milk for the normal dairy milk and the lactose-free milk (Lactaid in the US is the name brand, and it is usually available in 2% and skim varieties), so that fat content is controlled when comparing consistency. I recommend using 2% milk as it makes thicker yogurt than skim. It is also crucial that students let the warmed milk cool to 45°C prior to adding the starter. Hotter temperatures will kill the bacteria prior to fermentation. Proper heating initially (not boiling the milk) is also crucial – the heating alters the shape of the milk proteins and facilitates fermentation.

Incubator space can be a potential problem if this is being run with multiple lab sections. At the 2013 ABLE meeting at the University of Calgary, the laboratory staff was able to turn one of their cold-rooms into a warm-room, providing plenty of space for multiple beakers of fermenting milk.

Measuring Consistency of Milk and Yogurt.

Students should determine how they will measure consistency in the first week – have a large container of prepared (purchased or previously made in the lab) yogurt for them to test how their consistency methods will work once yogurt has set (*Do NOT purchase Greek yogurt for this purpose- it has been strained and is much thicker than any yogurt students will make). Most students will come up with some sort of drip-rate measure of consistency. Successful groups have used variations on the following methods:

- Upright spoon/stir rod method. Place a stir rod or spoon upright in the container of yogurt and time how long it takes the stir rod to fall to the side of the beaker.
- Pour/place a specified volume of milk/yogurt into a funnel lined with a cheese cloth and place funnel over a graduated cylinder.
- Determine the amount of time for "x" milliliters of fluid to drip through

- Count # number of drops and record time it takes to reach specified number (this works well for set yogurt, but the unfermented milk will pour through too quickly to count drops).
- Slope descent of drop of product – how long does it take a specified volume of product to descend a 45° slope? (Participants at the ABLE 2013 meeting covered a wooden block with aluminum foil, propped it at an angle and timed how long it took the milk and yogurt to descend the slope.)

Controls: Most students use heated and then cooled milk without any starter as a control, though occasionally they need prompting. One group asked if they could mimic the other ingredients in the yogurt starter without the bacteria, which is the proper control, but which is difficult to replicate without the proprietary formula that the starter manufacturer's use – though it is usually some combination of dried milk powder and dextrose. I usually then encourage them to do just one 50 ml sample as control for their cow's milk plus one 50 ml sample for their alternate milk product. If supplies or incubator space is a concern, the instructor can run a single cow's milk control and controls for the other milk products.

Interpreting Results for Cow's Milk: I have found results for cow's milk highly repeatable, though variation can result from the age of the milk and specific mix of bacteria in starter culture.

pH content of normal cow's milk yogurt: With proper background on the process of fermentation and the production of lactic acid, most students will predict that the pH of the yogurt will be lower than the starting milk, though it is important to emphasize this as a tangible connection to the metabolic process throughout the lab. Students also need to grasp that it is the changing acidity that contributes to the change in the protein structure of the milk and the thicker consistency. I have occasionally brought lemon juice and added it to plain cow's milk on the first week to show how the acidity changes the structure of the milk protein (and mention how this is one way to make cheese). Dilute (0.1 M) HCl could be used as well.

Monosaccharide content of cow's milk: Students have a harder time grasping the changes that occur in monosaccharide content. Lactose is a disaccharide and hence will not show up when tested with the test strips, which test for glucose and other monosaccharides. Normal dairy milk prior to fermentation will have negligible monosaccharide content. After fermentation, the relative monosaccharide content often increases -- the bacteria have broken apart most of the lactose into its constituent monosaccharides and consumed some of the monosaccharides, but not all. Occasionally, depending on culturing conditions, the bacteria will consume most of the monosaccharides, resulting in low monosaccharide content in the finished yogurt. This is a variable result, and instructors should be prepared to guide students through interpretations

of possible results.

Typical Results for Other Products: the pH will decrease after fermentation for all starting materials listed - evidence that there are fermentable sugars and that lactic acid is being produced. Even in products that don't have typical yogurt consistency, the pH decreases.

Lactaid/lactose reduced milk: Lactose reduced milk yields interesting results and **should always be included in this protocol**. A comparison of normal cow's milk with Lactaid/lactose reduced milk emphasizes the metabolic processes that are occurring in lactate fermentation – initial tests on Lactaid milk will show a high concentration of monosaccharides, which will then decrease somewhat in the fermented product. Students should grasp that the manufacturer of the Lactaid has added lactase enzyme that breaks the lactose disaccharide apart and that this is the reason for the higher glucose content in the Lactaid. I have found that there is little difference in consistency between Lactaid yogurt and normal dairy yogurt – though it is possible with a shorter incubation time, the Lactaid would set more quickly since the monosaccharides would be more readily available.

Plain unsweetened soy milk and coconut milk: These products will typically not set like typical yogurt, though there will be noticeable changes in consistency – often chunks floating in liquid. Students can search for recipes for soy milk and coconut milk yogurt on the internet and find that all suggest using sweetened soy milk, adding sugar to the starting unsweetened soy milk and coconut milk and usually adding a gelling agent like pectin or agar to improve consistency. Even with sufficient sugar added to the starting soy/coconut milk substrate, the proteins in these plant extracts are different than the proteins in cow's milk and do not form the same sort of gelled yogurt students are used to. It can be instructive to buy small containers of soy/coconut/almond milk commercial yogurt and have students examine the ingredients (and taste them outside of lab).

Sweetened soy milk: Sweetened soy milk yields a product that is recognizable as “yogurt”, but generally thinner than dairy yogurt. It will have a relatively high monosaccharide reading initially which will decrease after fermentation.

Almond milk: We often have difficulty finding unsweetened almond milk at the grocery store, and the sweetened almond milk ferments into something my students generally describe as “really gross looking”. Typically, a crust of thicker product forms on the top, liquid in the middle, and chunks that look like curdled milk toward the bottom. I have recently seen almond milk yogurt products in our local natural foods store, but these contain pectin, tapioca and other texture stabilizers.

Extensions: there are many possible extensions of this lab. The primary extension presented here is for students to com-

pare/contrast yogurt fermentation to alcoholic fermentation.

Kefir cultures: Kefir starter cultures can be purchased at many health food stores or ordered online – Yogourmet makes a kefir starter as well as a yogurt starter, and I've seen it in our large natural foods chain grocery store. Kefir cultures are a combination of yeast (alcoholic fermenters) and lactobacilli, and produce a beverage that is both a little bubbly from the carbon dioxide, has a little bit of ethanol content, and also contains lactate. The same laboratory activity can be done using the kefir cultures and the results compared.

Kombucha: kombucha is fermented sweet tea that is produced using a SCOBY (symbiotic culture of bacteria and yeast) and, like kefir, is thus a product of both lactate and alcoholic fermentation so it has both bubbles from carbon dioxide, lactic acid, and trace amounts of ethanol. Kombucha can be purchased in bottles at health food stores, and starter cultures can be acquired through internet-based sellers.

Making "Greek" yogurt: How do the many Greek yogurt products available differ from the plain yogurt made? Bring in plain yogurt and plain Greek yogurt and ask students to examine consistency, pH and glucose content (try to keep fat content constant).

Changing variables other than starting substrate: The effects of different temperature of initial heating, cooling, and incubation can also be easily investigated. Does putting the starter in when the milk is too hot kill the bacteria or slow down fermentation? What happens if cultures are incubated at 40 degrees?

Culturing bacteria from yogurt: There are multiple protocols in the literature for culturing the bacteria found in yogurt. BioRad makes a kit called "Microbes and Health Kit, What Causes Yogurtiness? Biotechnology Explorer" Product # 166-5030EDU –it is available for purchase and has instructions and materials for culturing yogurt bacteria (\$121 for supplies for 32 students).

*Student tasting of yogurt outside the laboratory:*** **Safety note:** all tasting of purchased yogurt is done outside of the lab room and the yogurt for testing is stored in the faculty/staff lunch refrigerator and not in the lab refrigerators. If possible, bring in plain commercially available yogurt and disposable spoons for students to taste safely outside of the lab area. Students enjoy this, and many have never tasted unsweetened yogurt before. Commercial soy yogurt is readily available in larger containers, and it makes an interesting comparison. I have also brought in kefir samples as well and asked students to compare yogurt and kefir – the carbonation is detectable in the kefir.

Evaluation: This lab is generally used as the basis for a full lab report. After the initial week, I have students write up the

methods section and the initial results (monosaccharide content, pH and consistency) from the unfermented milk and turn it in at the beginning of the second lab period. They get feedback on the methods and part of the results sections prior to submitting the whole paper. Expectations for background research and understanding of biochemistry can be adjusted to the level of the students in the course.

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