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Bringing Biochemistry Home: Transforming Milk into Yogurt

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BRINGING BIOCHEMISTRY HOME: TRANSFORMING MILK INTO YOGURT

By

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A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

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I would like to first and foremost acknowledge Dr. Susan Pedigo, my gracious thesis advisor, professor, and companion for all her time and effort spent on this project. She constructed the idea for this project, working around COVID-19 restrictions and coming up with a lab that could be done in the students' kitchens. The pandemic created difficulties that the majority of students and teachers had not faced before, but Dr. Pedigo did not let any of these hinder the progress of our experiment. I encountered my own personal challenges of taking the DAT, applying for dental school, and family and health related issues, but she always kept me on track and constantly supported me. She helped me create sufficient deadlines, set up biweekly meetings to ensure my progress, and assisted me in any way she could. Without her initiative, patience, and assistance this project would not have been possible. Thank you Dr. Pedigo, not only for supporting me throughout this project, but for all four years I spent at Ole Miss. Your dedication to all of your students, especially myself, is inspiring. I would also like to thank my second and third readers, Dr. Wayne Gray and Dr. John Rimoldi for sacrificing their own time to support me. I am very grateful for your insight on this project and appreciate your responsiveness. Lastly, I would like to thank Skylar Nash, a fellow peer of mine in the honors college who helped initiate this experiment.

ABSTRACT

Ashley King: BRINGING BIOCHEMISTRY HOME: TRANSFORMING MILK INTO YOGURT

Communicating the beauty and complexity of biochemistry to students in a large classroom during the pandemic: what a challenge! We undertook a novel endeavor in the Department of Chemistry and Biochemistry by introducing a mandatory kitchen chemistry experiment in a lecture course. Milk, the epitome of our identity as mammals, also contains all of the major biochemical macromolecules studied in Biochemistry I. Further, the making of yogurt invokes physical processes that are the major processes and molecular forces that dominate the content of the course. Here, we report the results of massive parallel experiment conducted in the kitchens of the students enrolled in a large lecture course in our department at the University of Mississippi. Students prepared yogurt from kits that we supplied. Our hypotheses were: 1) lactose was required for curd formation, so lactose free milk would not form curd; 2) Only casein containing milk would form curds, so soy milk would not form curd; and 3) Fermentation by the bacterial cultures would cause the yogurt to be acidic relative to the milk from which it was made. The two measurable quantities were the pH of the solution and an informal viscosity measurement performed by dropping a stainless-steel ball through a standardized column on yogurt (BB drop test). Thus, students were assigned a control milk and a test milk of five types: Whole, 2%, Fat Free, Lactose Free, and Soy. An online form was developed for students to submit all relevant aspects of their “lab report. A total of 127 complete reports were submitted. An analysis was done to determine the relevance of the recorded information, resulting in the deletion of nine entries. Formats of entries were

standardized. Results showed that 1) Lactose free milk formed curds. 2) Soy milk formed a very thin curd, but thickened. 3) All milks were acidified after incubation in the bacterial starter culture. Suggestions for revision of protocol and reporting of the experiment are provided.

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CHAPTER 1

INTRODUCTION

Milk is the exclusive source for mammals to gain necessary calcium, vitamins, and carbon-based nutrients, and is the only source of these nutrients for newborns. As many things in our lives that are ordinary, we fail to recognize how extraordinary they are. Milk is water that based on its function, is appropriately populated with a variety of nutrients. It is an aqueous suspension of protein particles, a solution of soluble carbohydrate and proteins, and an emulsion of fat droplets. As such, all categories of major carbon nutrients are provided along with micronutrients to ensure the success of infant mammals until they can manage to eat on their own. Female mammals are engineered with a specialized exocrine gland to produce this material- ordinary and extraordinary- at the same time.

To create a new, fully functional individual, the carbons in milk are provided as the building blocks in the form of carbohydrates, proteins, and fats in the form of triglycerides. Each of these components is balanced in terms of solubility, energy density and functional form for its utility as a building block for growth. **Table 1** below provides the distribution of nutrients in cow milk. Whey and casein are the protein components in mammalian milk. Whey is a mixture of soluble, globular proteins that remain in the aqueous phase when milk "curdles". Casein, on the other hand, is an aggregate of intrinsically several disordered proteins that has neutral buoyancy in water, but it is so large it scatters light. Casein is the component of milk that forms the curd during the process of yogurt making. The carbohydrate in mammalian milk is lactose, a disaccharide. Milk fat is a molecule of glycerol that is esterified with 3 fatty acids, or a triglyceride.

The fatty acid composition of mammalian milk controls its physical characteristics as a function of temperature. As an animal product, milk fat comprised of primarily saturated fatty acids (35% long chain, 25% short chain, and 40% unsaturated),[1] leading separated milk fat or butter to be a soft solid at room temperature.

Table 1: Percent by weight in cow milk[2]

Component	**Percent by weight**
Water	87.5
Casein and Whey Proteins	3.4 (80:20)[3]
Lactose	4.8
Milk Fat	3.9
Minerals (Ash)	0.8

Milk is white. Why? It is because it is both an emulsion and a suspension. The emulsion is due to milk fat. As it is made in a mammal, milk is at 37 °C. At that temperature milk fat will be a liquid, but insoluble in the aqueous phase of milk. The solubilize milk fat droplets formed in the mammary glands, they are coated in a single layer of phosphoacylglycerol, and then immediately before secretion in an additional lipid bilayer as they are released into the lobules of the mammary gland. Thus, the triglycerides of milk fat are suspended in the aqueous phase by virtue of favorable interactions between phosphatidic acid headgroups of the packaging with water. Milk fat has lower density than water, so the milk fat droplets float if the milk has not been homogenized. Milk is a suspension because the casein particles are essentially aggregates of intrinsically disordered proteins that are so large they scatter visible light. These protein aggregates are assembled in the Golgi are secreted from there directly into the lumen of the mammary gland lobules. Under normal circumstances, casein particles do not form larger aggregates due electrostatic repulsion due to the excess of negative charges on the casein subtypes that comprise the casein particles. Milk is white because the casein particles and the milk fat droplets are so large that they scatter visible light. It

is common experience that low fat milk looks white, but "watered-down" relative to whole milk. That effect is due to the decrease in scattering of visible light resulting from the loss of fat droplets in fat free milk.

The delivery of calcium and casein structure and function are integrally intertwined. In general, casein particles are micellar-type structures with hydrophilic groups on the outside in contact with water. These unstructured casein components are three major components: α- casein, β-casein, and κ-casein that are held together through hydrophobic interactions and through phosphorylation of the polypeptide to form sites for chelation of calcium and calcium phosphates.[4] Each of these caseins has different lengths and a different number of phosphorylation sites (bovine α- casein (s1(119 aa)-8 sites, s2 (159 aa)- 11-13 sites, β-casein (44 aa) – 5 sites), and κ-casein (153 aa)- 1 site).[4] These interactions between calcium and phosphate hold the casein components together, and create a density of calcium that exceeds its solubility in the solution on its own, thereby preventing formation of calcium phosphate crystals, a nightmarish needle-like structure that would damage both mother and child.[5] The phosphorylation sites are often directly adjacent or closely positioned so that there are multiple contributions to chelation of calcium from a single polypeptide chain.[4] While the calcium-phosphate interactions are in the interior portion of the casein "micelles," while the disordered casein protein portions protrude from the calcium-phosphate core in formations often illustrated in shapes similar to spaghetti noodles since they cannot otherwise be visualized (**Figure 1**). κ-casein is different from the other two casein components in that it has only one phosphorylation site. The distribution of its amino acids is asymmetric with its basic and hydrophobic residues in the N-terminal region of the molecule, which is buried in the core structure of the casein particle. The "spaghetti" is comprised of the remaining ~ third of κ-casein with a predominance of acidic residues that protrude from the core

structure. The disordered surface components repel each other because they are highly negative.[5] Casein particles will coagulate to form a curd in milk when the solution becomes acidic, because protonation of the acidic groups neutralizes them and so electrostatic repulsion is decreased. The acidity levels that cause the casein particles to aggregate is a result of anaerobic glycolysis, as will be discussed below.

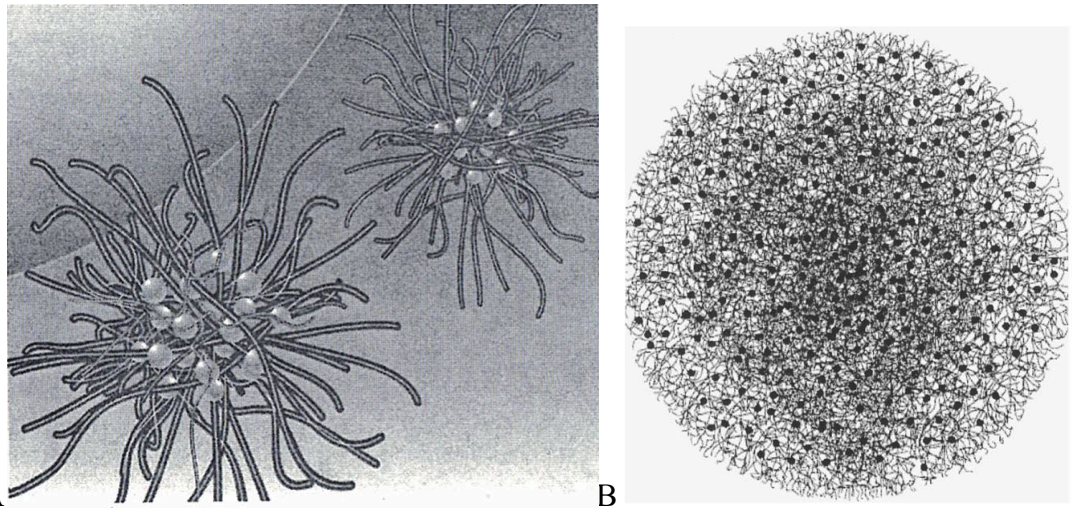


Figure 1: Two representations of casein particles.

(A) Spaghetti representation illustrating the unstructured nature of the C-terminal tails of κ-casein.[6] The spheres in the center represent calcium ions chelated by the phosphorylated caseins. (B) A plum-pudding representation of a casein particle.[7] The furry outer surface is comprised of the tails of κ-casein.

Lactose is a disaccharide, meaning that it is made up of two number of monosaccharide units, which are the basic units of carbohydrates. Lactose is a disaccharide made up of Galactose and Glucose bonded together through a β-1,4-glycosidic linkage. Glucose and Galactose are C-4 epimers, which means they are structurally identical except that they have opposite chirality at C-4. A glycosidic linkage is a covalent bond between two monosaccharides and in the case of lactose, the β-1,4 linkage refers to the configuration of the anomeric carbon and its bond the other monosaccharide. This linkage is between the anomeric carbon on β-D-galactose and the hydroxyl

oxygen atom on C-4 of the β-D-glucose. When humans ingest lactose, the hydrolase intestinal lactase cleaves the β-1,4-glycosidic linkage in lactose to form free glucose and galactose. These molecules are the moved through the wall of the enterocytes with the Na+-Glucose transporter, and then out into the extracellular space by a GLUT2 transporter to be picked up by the blood and circulated to the tissues.[1] Glucose and Galactose both pass down gradient through GLUT transporters into tissues. Now that they are in the tissues, they must be degraded to produce ATP, or stored as Glycogen as the tissues require. In the case of aerobic catabolism in our cells, glucose is fed directly into glycolysis. The galactose molecule is converted into glucose via three unique steps catalyzed by galactokinase, transferase and epimerase, and then fed into glycolysis as a glucose molecule. The glucose molecules are converted into pyruvate during glycolysis.

For the organisms in yogurt, the story is different. As yogurt forms, there is little oxygen on the interior of the culture, so the pyruvate will be reduced to lactate by NADH through lactic acid fermentation, catalyzed by the enzyme lactate dehydrogenase. The reason that the bacteria must do this is that the reducing agent NADH must be oxidized to NAD^+ which feeds back into glycolysis to perpetuate the glycolytic cycle. Lactate is secreted from cells as lactic acid and so acidified the solution in which it forms. The acid causes protonation of the κ-casein and neutralizes the negatively charged surface of the casein particles and making them more hydrophobic. The hydrophobic interaction forces in the aqueous milk solution drive the neutralized casein particles together as they bury their now hydrophobic surfaces, and curds form. This formation of curds facilitates the synthesis of yogurt as the solution becomes more viscous, and acidic.

Yogurt is made by combining milk heated to approximately 113°F (43°C) with yogurt culture that contains Lactobacillus bulgaricus and Streptococcus thermophilus. Lactobacillus bulgaricus is a lactic-acid producing, rod shaped bacillus with a genome that indicates ongoing

specialization. Streptococcus thermophilus is a fermentative facultative anaerobe, gram positive, coccus (sphere-shaped) bacterium. Streptococcus thermophilus uses amino acids synthesized by Lactobacillus bulgaricus to produce lactic acid, lowering the pH and optimizing Lactobacillus bulgaricus growth. Lactobacillus bulgaricus reduces the pH further by generating more lactic acid through fermentation. Both of the bacteria consume lactose as a form of energy to facilitate lactic acid fermentation.[8]

CHAPTER 2

PROTOCOL DEVELOPMENT AND HYPOTHESES

The conception of this experiment emerged from a realization that yogurt making from cow's milk encompasses many of the major concepts of first semester biochemistry (Chem 471) including: pH and protonation states of amino acids, protein folding and noncovalent forces, triglycerides and their packaging for water solubility and disaccharides chemistry. In the process of making yogurt, these topics are coupled with the basics of anaerobic metabolism, which directly couples the basics of biological molecules described above to the basics of anaerobic processing or fermentation of pyruvate to lactate. Further, the kitchen chemistry aspect of this project engages the knowledge of the classroom into the home experience of the students in their very own kitchens.

To execute this project, Skyler Nash, Dr. Pedigo and I worked through all aspects of the experimental protocol, with careful consideration of what equipment would be available to a typical student. We also considered what aspects of the experiment we could control, and the measurements that would be made on the yogurt to assess its properties. In addition, we wanted to focus on the lactose and casein components of milk and their contribution to formation of the yogurt. To this end, the experiment examined the various milks and yogurt based on their chemical makeup. Whole milk, 2%, and fat free milk were chosen to represent cow's milk, each containing casein and lactose, but with differing levels of milk fat. Soy milk and lactose free milk were tested to examine if yogurt would form from milks lacking essential components in the formation of yogurt. Lactose-free milk lacks lactose, obviously, but still has casein. Soy milk lacks casein as

well as lactose. My hypothesis is that the soy and lactose free milks will not form yogurt because they do not contain essential components, lactose or casein.

We needed to consider what were the two major aspects of the yogurt that we could measure in a take-home chemistry kit. First, we know that curds should form to thicken the yogurt. The more the particles aggregate and form curds, the more the yogurt thickens and the viscosity increases. In order to measure the viscosity, we decided to create a test based on the density measurement of ethylene glycol in radiator fluid. We measured the time a BB pellet takes to reach the bottom of a 50 mL conical for both the milk and the yogurt culture. The increase in time for the bead to fall is a qualitative indication of the viscosity of the curd formation. Second, anaerobic (and aerobic) glycolysis should acidify the solution. Thus, a pH test was designed on the milk and yogurt to indicate if there has been a chemical change due to bacterial activity.

This class wide experiment analyzes the results of these two tests on the five different types of milk. To isolate the measurables noted in the previous paragraph, the rest of the data must be controlled. Kits for the 127 students were assembled with 4 pH strips, a yogurt starter culture, a thermometer, two 50 mL conical vials, and BB pellets. The purpose of the conicals was to ensure that all students had the standardized path length for the BB drop tests. The kits were marked with a color. Each corresponds with a combination of milks (Control: Test) that the student would be using: pink is whole milk and 2%, green is whole milk and fat free milk, orange is whole milk and lactose free milk, and purple is 2% and soy milk. The 2% control was used with the soy milk because the fat content of soy milk is less than 3%. A protocol was crafted to distribute to the students with instructions on how to make the yogurt using the purchased milk combined with the provided yogurt starter culture. The complete protocol is in the **APPENDIX**.

The purpose of the protocol is to guide the students and ensure the data were being controlled for quality results. The protocol instructed students to boil two cups of milk while stirring intermittently and to cool the milk to 100°F before adding the culture, then incubate the growing culture in an oven that started warm, then cooled. After 10 hours, the culture can be stored in the refrigerator until ready to perform the experiment (BB drop test and pH measurement) or tested immediately upon completing the incubation. Importantly, a google form was also created as a place for students to record all aspects of their materials and experimental conditions, and to submit their results from the experiment. Students uploaded photos of the experiments being conducted from their homes, and often added comments about observations. **Figure 2** shows examples of two photos that students attached.

A   B

Figure 2: Photos from student submissions to the Google Form lab report.

(A) Stove-top and (B) microwave heating of milk prior to culture addition.

The first few kits were distributed and students were unable to form yogurt from the culture given (Activia). One student's mother was able to cultivate yogurt in her incubator where she regularly makes yogurt. Activia yogurt is advertised as an active culture yogurt, but was extremely faulty in practice except under ideal conditions. We were able to "re-purpose" these otherwise useless starter cultures at the Girls and Boys Club. For the class, yogurt starter culture was replaced

with a few tablespoons of Great Value Plain Yogurt from Walmart (in a baggie), and distributed to all students in their kits. Further complications followed. The supplier did not send enough thermometers, limiting the number of kits that could be initially distributed. The pandemic prevented class from meeting in person, so kits were handed out by me in the grove with my three-month-old puppy. Once the second shipment of thermometers came in, more kits were distributed from the lab in Coulter. There was not enough yogurt culture for the last few dozen kits, so more was made from a yogurt culture that was made from the Great Value Plain Yogurt. Thirteen students were not resident in Oxford, and required kits to be mailed which delayed their experiments. The unconventional academic semester coupled with the delays in protocol and kit development meant that the launching of the kitchen chemistry experiment was completed by November 12, 2020.

The experiments were conducted, and the data were submitted online. As students began turning in their data, concerns were raised about the BB drop test. The pellets were not reaching the bottom of the conicals for some students. This result may be a lack of recognition when the BB actually hit the bottom of the tube, or it may have been real and correlate with increased time spent in the refrigerator. The importance of the time spent in the refrigerator was not initially considered, and could have been another factor that should have been controlled. At this point I included an entry in the Google Form regarding the time in the refrigerator and out of the refrigerator relative to the BB drop test. The BB drop test and the pH tests were recorded by students through the google form. Students had limitations in their schedules, so the timing of these incubation periods is a problematic aspect of this kitchen chemistry experiment in general.

CHAPTER 3

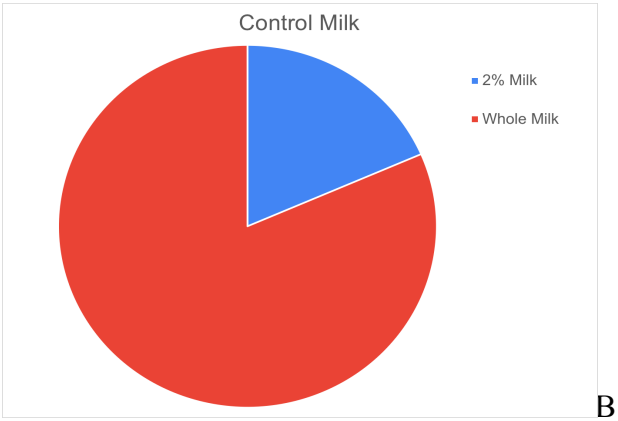
DATA

This experiment was conducted by two sections of Dr. Pedigo's Chemistry 471 class, 127 students total. Upon consolidating the data and analyzing the results, eight of the students' results were considered unusable because of their submission on the google form submission. The document asked students how long the BB pellet took to reach the bottom of the conical, and these eight students submitted answers without any units and therefore had to be thrown out, leaving 119 available submissions to compare. Each student was assigned two different kinds of milk, a control and a test milk. The possible assignments are below:

Table 2: The pairing of the control and test milks

CONTROL	**TEST**
Whole Cow's Milk	2 % Cow's Milk
Whole Cow's Milk	Fat Free Cow's Milk
Whole Cow's Milk	Whole Cow's Milk-LACTOSE-free
2 % Cow's Milk	SOY Milk -unsweetened

The graphs in **Figure 3** represent the percentage of students assigned to each milk. The control milk data are shown on the right and the test milk is on the left.



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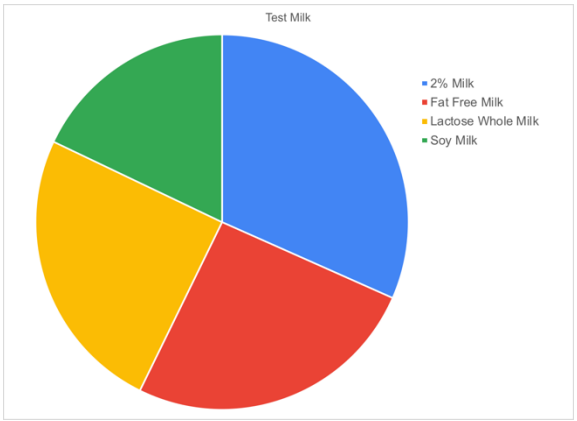

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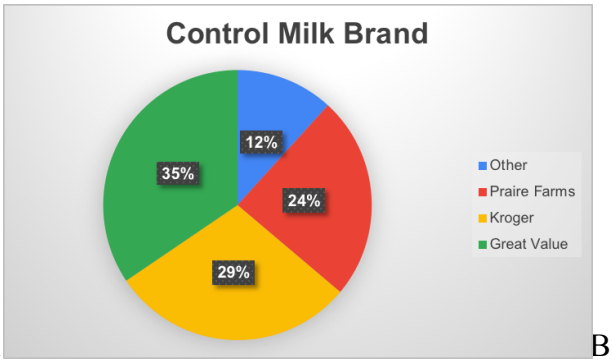
Figure 3: Pie charts illustrating the control (A) and test (B) milk distributions.
The 2% milk in the control set was for the Soy milk in the test group.

The types of milk were controlled, but the brands of milk varied between each student. The variance in brand was recorded by the students but did not have a significant effect on the results because different brands of milks are comprised of generally the same macromolecules. Below are the brands of milk used by the students (**Figure 4**). The graph on the left represents the control milk, either whole or 2%. The graph on the right represents the test milk, either whole, 2%, soy, or lactose free.

A 

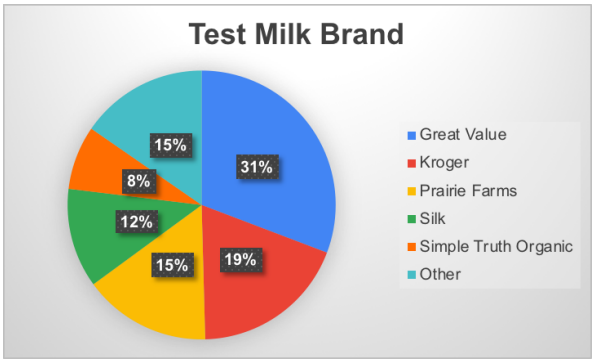
B 

Figure 4: Brands for the control (A) and test (B) milks.

In order to execute the experiment according to the protocol, students had to heat their milk before cooling it and adding yogurt culture. Since the experiments were conducted in the students' kitchens, a majority of them heated their milk on the stove. Some of the students had limited or no access to a kitchen and had to heat their milk in the microwave. The number of students is depicted on the graph below (**Figure 5**). The source used to heat the control and test milks was the same.

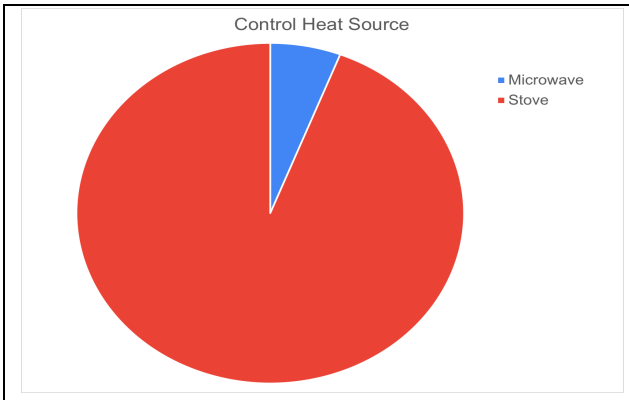
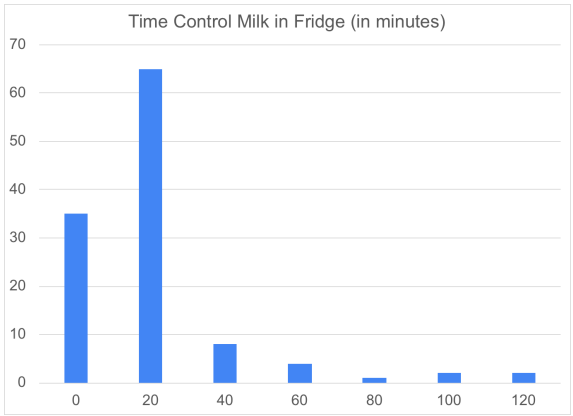


Figure 5: Stoves vs. Microwaves.

Pie chart depicting the number of students with access to stoves and to microwaves for the initial heating of the milks prior to culturing.

Students were given the choice to leave the yogurt in the fridge before running the BB pellet drop tests and the pH tests. Initially, we did not consider the time in the fridge to be an important factor in this experiment, but as students began attempting the pellet drop test, we began getting complaints that the BB never reached the bottom of the conical. We hypothesized that the time left in the fridge may correlate with the drop time for the BB. A spot on the google form was added for students to record how long they left their yogurt in the fridge after incubating the culture, and how long they left it out of the fridge before they ran the BB drop and pH tests (**Figure 6**).



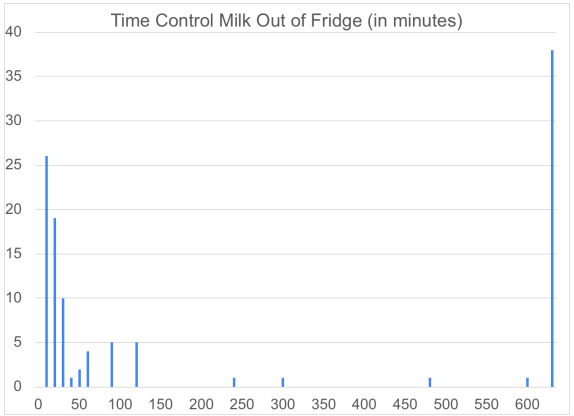


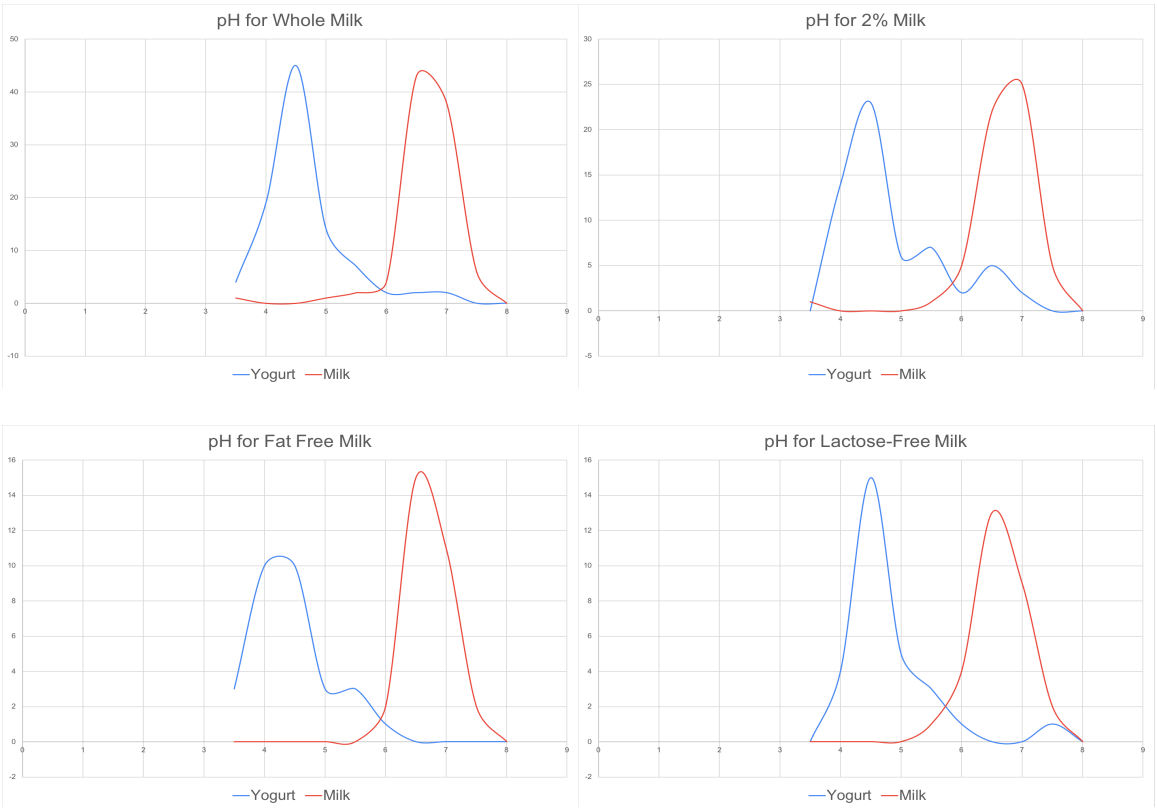
Figure 6: Considering the incubation temperature for the yogurt before measurement.
The left figure is the time at 4°C after culturing. The right figure is the time at room temperature before measuring the pH and BB drop time on the yogurt cultures.

The 38 people that kept the culture at room temperature for 10 hours before they made the measurements likely never cooled the cultures in the refrigerator since the protocol called for the cultures to be incubated at room temperature, essentially, for the 10-hour growth period of the cultures. We suspect that is the time that they recorded there.

CHAPTER 4

RESULTS

Tests were conducted to analyze the pH and the viscosity of both the yogurt and the milk. The pH tests were conducted using strips from the kits provided. A photo of the pH scale was posted on the protocol for students to compare their results and determine the pH of their products. The results were collected on the google form and compared on an excel sheet. The frequency of each pH value is plotted in **Figure R**, one for each type of milk tested. The average pH and standard deviation for each milk and the yogurt sample is reported in **Table R**.



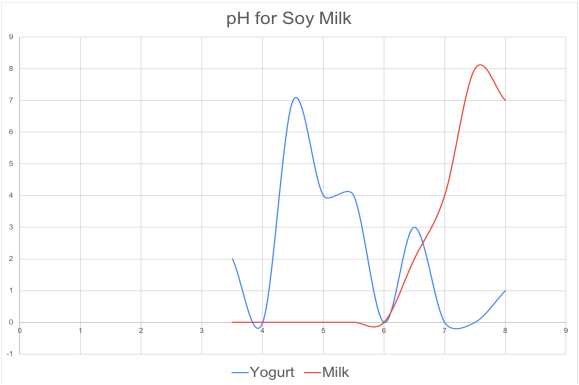


Figure 7: Graphical representation of the cumulative pH values for the test and control yogurts (blue) and milks (red). The x-axis represents the pH and the y-axis represents the number of students responding.

Table 3: The average and standard deviation in the average for the pHs of the milks and yogurts.

Type	Average Yogurt pH	Stdev Yogurt pH	Average Milk pH	Stdev Milk pH
Fat Free Milk	4.4	0.6	6.7	0.4
2% Milk	4.9	0.8	6.7	0.6
Whole Milk	4.6	0.7	6.7	0.6
Lactose-free Whole Milk	4.8	0.7	6.6	0.4
Soy Milk	5.1	1.0	7.5	0.5

All milks became more acidic after inoculation with bacterial culture and incubation. These data represent that all of the milks from cows started and ended with similar pH values. The soy milk was more basic than the cow milk to start with by almost a full pH unit. After a 10 hours incubation with a bacterial culture in it, the soy yogurt culture then had a significantly less acidic pH at 5.1. Thus, the bacterial growth and utilization of nutrients dropped the pH by 2.3 units in soy milk. The only cow milk to see an equivalent drop was fat free milk.

The second test conducted to determine the viscosity of the yogurt was the BB pellet drop test. Each student dropped two BB into a 50 mL conical containing their yogurt and measured the time each took for it to reach the bottom. A BB drop test was run on each of the milk samples as well, but they were all relatively uniform in reporting that the BB reached the bottom of the milk instantaneously (1-3 seconds). We used this value as the “blank” of the yogurt test and essentially

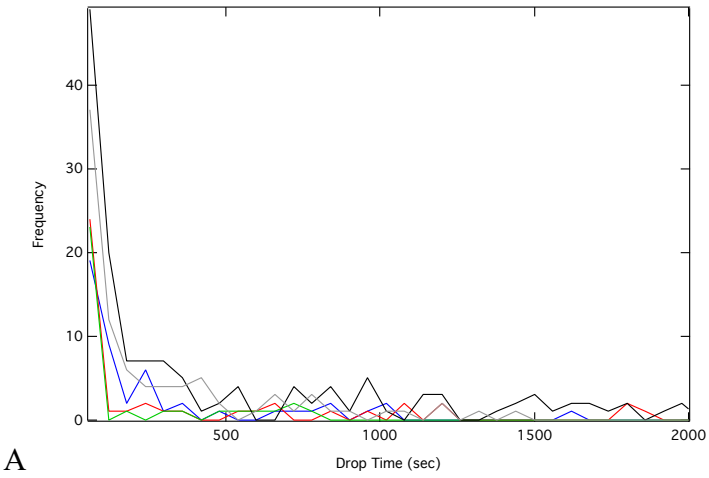
neglected all data that reported the BB drop time at less than 5 seconds. Second, data were binned by milk type, and sorted from long drop times to short times. The short drop times were trimmed as mentioned above based on the drop time in uncultured milk. For consideration of outliers for the data set at long drop times, we used the Q-test according to the equation below.[9]

$$\frac{(x_{hi} - x_{hi-1})}{(x_{hi} - x_{lo})} = Q$$

The value of Q must be greater than 0.4 for 10 data points. In our case the smallest data set with the greatest stringency restriction was 31 data points, and the Q value was 0.4. All other Q values were significantly larger (0.5 to 0.8). The average and standard deviation in the average drop time for the optimized data sets are reported in **Table S** below. The graphs in **Figure S** show the data for each of the five milks. The average Drop time for the whole milk is significantly greater than the milks. However, the population of the is significantly greater. Is the effect we see due to just a larger sample size? Statistically this is hard to predict. One interesting indication that we can roughly group our Fat Free, 2% and Whole cow milks together in a group is found in Figure SC.

Table 4 The average and standard deviation for the time in the BB drop test of yogurts.

MILK	Min value	Max value	Ave Time (sec)	Std Time (sec)	Median Time (sec)	Number of Points
2% Milk	5	1425	280	343	120	95
Fat Free Milk	5	1620	287	403	120	51
Lactose Free Milk	8	1869	369	542	31	38
Soy Milk	5	780	167	251	16	31
Whole Milk	5	7200	566	786	210	153



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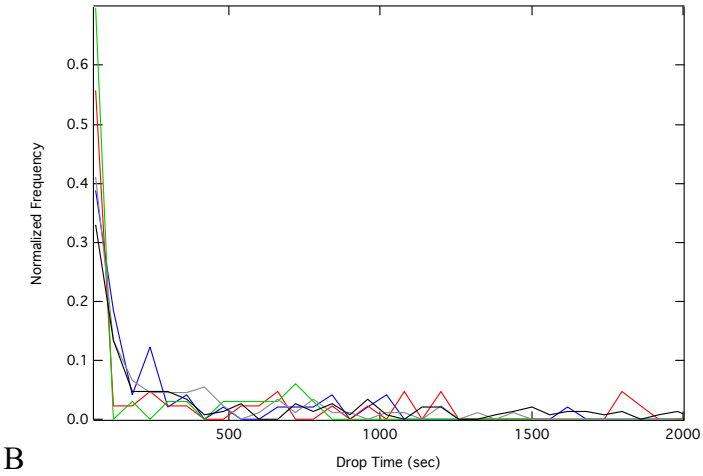

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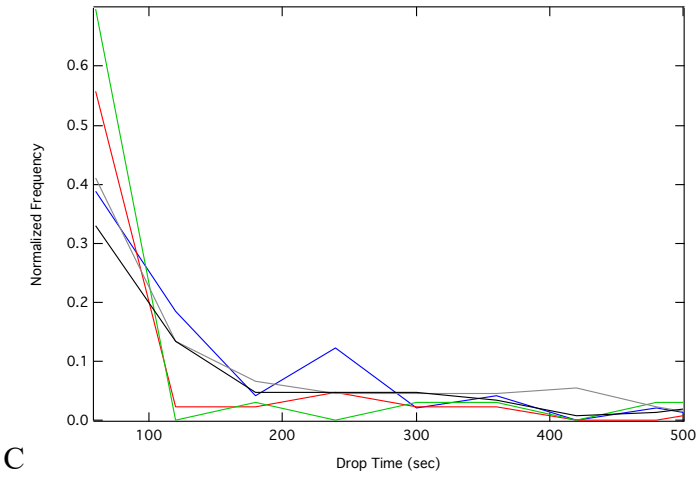

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Figure 8. Plots of the BB Drop Test data.

(A) The Whole (black), 2% (gray), Fat Free (blue), Lactose Free (red) and Soy (green) milk are all plotted against their absolute frequencies. The bin size is 60 seconds. (B) Here the same data are normalized against the data set size to highlight the inherent trends in the data. (C) Replot of Figure B to highlight the Y-axis values for the normalized frequency data.

Figure 8C highlights the high frequency of short drop times in the lactose free and soy milks and the relative similarity and low frequency of the 1-minute drop time for the Fat Free, 2% and Whole milks. This trend appears to be the systematic source of the difference in the average and median drop times for the two groups.

CHAPTER 5

DISCUSSION

Upon analyzing the data, we were able to determine key information pertaining to our hypotheses. Each of the graphs representing the pH of the milks and yogurts showed a consistent decrease in pH across all milks when yogurt was created, implying that the bacteria in each milk had a carbohydrate to consume. The cow milk, just as we had predicted, successfully created yogurt and caused a decrease in pH. We hypothesized that the soymilk and lactose free milk would not have a pH decrease because neither milk contains lactose for the bacteria to consume and therefore, we did not expect the solution to become acidic. While neither milk does possess lactose, they were able to form yogurt proving that the bacteria relied on other carbohydrates. We will discuss each of these in turn.

Regarding lactose free milk, we assumed that it not only does not contain lactose, it doesn't contain sugar for the bacteria to eat. Neither of these is true. Since lactose free milk utilizes the enzyme lactase to break down the disaccharide lactose into the simple sugars, glucose, and galactose, those sugars and residual lactose remain in lactose free milk. All of these sugars are then available to the bacteria to eat in anaerobic glycolysis, therefore acidifying the solution and lowering the pH. Analyzing the results further, we noticed that in **Table 3**, there is a correlation between pH of the milks that contain casein and the yogurt that they create. Fat free, 2%, and whole milk had average pH values of 6.7 and the average pH for lactose free milk was 6.6. The average pH of yogurt for all four milks was 4.675. The average pH for soymilk was 7.5 and for the yogurt created the pH was 5.1. The pH difference between milks with casein and the milk

without is one unit, meaning the [H^+] of the casein milks was ten times larger than for the soymilk. Researching further we found that the pI of casein is 4.6, which is likely the reason for the pH drop in the cow's milk to that pH. The casein could be acting as a pH buffering agent, buffering the protons and ultimately dominating the solution's pH. That would explain why the pH for the yogurts containing casein averaged very close to the pH of casein and why the one milk that does not contain casein has higher pH values. Further, the implication is that milk itself is a poor buffer, and the protons released from the fermentation process lower the pH of the solution, but only to the pI of casein. Whether there are lots of protons released in the situation of high levels of lactose or low levels of protons, with lower levels of lactose or free sugar, the effect on the pH would be the same- pH is equal to the pI of the buffering agent- casein.

Soymilk does not contain casein, but soybeans do contain fiber and simple sugar. The nutrient label from Silk brand Unsweetened Soy Milk is included in the Appendix. It has only 4 g carbohydrates in total, a value that is about 20% less than the carbohydrates in cow milk. However, notice that the majority of those are indigestible and only 1 g of the total carbohydrate is Glucose. We originally hypothesized that glucose could be consumed by the bacteria thus making CO_2 and lactic acid and lowering the pH. While we were researching information to explain the thickening of the yogurt, we found that soymilk contains the proteins of β-conglycinin and glycinin which both have a pI of approximately 5.5. These proteins could be dominating the solution and driving the pH down just as we hypothesized the casein is in the other four milks. Our data predict that the actual pI of the glycinins are closer to 5.1 than 5.5, and that the glucose found in soymilk is indeed being digested to lactic acid by the bacteria.

The BB pellet drop test was utilized to determine whether or not the milk formed curd while carrying out the experiment to make yogurt. We hypothesized that the curd formation would

be dependent on the presence of casein and our results correlated with this proposition. Out of all five of the milk samples, soymilk was the least dense and therefore formed the least amount of curds. Soymilk was the only milk that we tested that did not contain casein, the aggregating protein present in the other milks. We hypothesized that there would be no curds formed, but the results displayed there were minute curds formed and the particles did coagulate slightly. The reason for this may be because of the amino acid sequences of β-conglycinin and glycinin. The decrease in pH of the solution measured in the pH test above would cause protonation of the glycinin proteins. Since their pI is acidic, and fermentation acidified the solution to their pI, protonation would neutralize them and trigger aggregation and the particle formation. In industrial processes, this process is promoted by driving the particles together even more with the addition of Glucono-δ-lactone, which is used to make tofu from soy milk.[10] Gluconolactone further acidifies the solution and causes the solution to curdle. Some students commented that the yogurt made from soymilk was slimy, and this would correspond with the thought that β-conglycinin and glycinin would cause the particles to aggregate, but not form serious curds.

Will there be a next time?

There was room for improvement in this experiment that became evident upon reflection. In regard to the data collection, there were a number of changes to the google form that would make data analysis easier. Recording of student name should have been standardized (Last, First). The Google Form could have been formatted so that there was a drop-down option to select units, avoiding repetitive and unclear submissions. Questions to which students would all have the same answers would be eliminated. The brand of milk was not relevant data to include for this experiment and could have been omitted, although if further analyzed the difference brands could

have an impact based on their chemical makeups. This would have made the data analysis much easier and less time consuming. In regard to the experiment itself, the factors that we controlled seemed to be vital to reaching the conclusions that we did. For more accurate data, we could have specified exact incubation times for heating and cooling before measurements. We could have taken into account the length of the conical in relation to the BB drop time. A longer conical could have been used to lower the opportunity for error. The stopwatch method for timing the BB was not perfectly precise but working from home limited the options for that part of the experiment. Students could have conducted the experiment with more than two types of milks to increase the amount of data to interpret and analyze. Our inoculation culture needed to be properly standardized. We probably needed to buy it, homogenize it, and to carefully measure the amount that each student should add to their heated milk. Finally, the entire experiment would be facilitated by face-to-face contact with the students. If we had been able to distribute milk samples, it would have reduced the financial burden. Perhaps we could have conducted the lab in a teaching lab space in Coulter Hall.

Concluding Remarks:

This experiment was ambitious and exhausting, but the students seemed to really appreciate the opportunity to do a hands-on exercise that reinforced ideas that we learned in a classroom setting.

APPENDIX

1. Protocol
2. Nutrient label for Soy Milk

Yogurt Lab Protocol

Each student will be assigned a Control and a Test sample. This lab occurs in two steps. First, make the yogurt (heat milk, add culture, incubate). Then second, make measurements on the yogurt (pH and viscosity). Record all results and pics into the Lab Report Google Form.

DATA SETS:

GROUP	CONTROL	TEST
PINK	Whole Cow's Milk	2 % Cow's Milk
GREEN	Whole Cow's Milk	Fat Free Cow's Milk
ORANGE	Whole Cow's Milk	Whole Cow's Milk-LACTOSE-free
PURPLE	2 % Cow's Milk	SOY Milk -unsweetened

Note: Please save your receipt for the milk, and upload it in the Lab Report Google Form. We will look for a way to reimburse you if we possibly can.

Materials:

1. Control Milk and Test Milk as per your assignment.
   (Suggestion: 1 qt milk of each kind- just in case you need extra)
2. Pot for heating milk on stove -or- microwave-safe pot for microwave
3. Measuring cup
4. Tablespoon spoon
5. Yogurt culture (provided- except for mailed kits)
6. Spoon for stirring milk as it heats
7. Thermometer (provided)
8. Glass cup or bowl (for after culturing yogurt)
9. Saran Wrap or foil
10. pH paper (4 sheets; provided)
11. 2 x 50 mL conicals (provided)
12. Transfer pipets (provided)
13. BBs in small microfuge tube (provided)

Procedure:

Step 1: Making Yogurt
(One pot prep time: 2 hours)
(Two pot prep time: 1 hour 15 min)

1. Measure 2 cups of your designated milk into the pot (**Figure 1)**
2. Start the timer. Heat on medium heat with **constant stirring** until milk just starts to boil. (**Figure 2** &3; ~10 min)***

CAUTION: Milk must be stirred constantly or it will burn to the bottom of the pot. As soon as it starts to boil remove from heat immediately. It will boil over very quickly.

3. Remove from heat, and cool milk to 100 degrees F (stirring will encourage the milk to cool).
4. Add 1 tablespoon of the yogurt culture (**Figure 4**), stir well to make sure the yogurt is evenly distributed.
5. Place the covering over the glass container (**Figures 5 & 6**). Put the glass containers (Control and Test) on a plate or tray.

6. Preheat the oven on **LOW** for 5 minutes. Turn the oven off. Put the tray in the oven with no additional heat added other than the warm oven.
7. Remove the yogurt after 10 hours. Place glass containers in the refrigerator until you perform Step 2.

*** Microwave instructions: Heat milk until it is boiling (~ 3 minutes, depending on power)

Step 2: Measurements of Properties
(Total Time: 20 minutes)

1. In each measurement, you need to make the measurement on milk, and the yogurt that is made from it. Since you have a Control and a Test milk, you will have a Control and Test yogurt- so 4 total samples for each measurement.
2. Assess the pH Milks and Yogurts- only one measurement for each of 4 samples.
   a. Use the transfer pipet to test the pH of Control and Test milks and yogurts
   b. Suggestion: Touch the edge of the pH paper against the liquid or the yogurt rather than dipping it in. (See **Figure 7**.)
   c. The Key for the pH paper is shown in **Figure 8** below.

(Note: The pH test can be performed on milk and yogurt samples that are in the conicals awaiting the BB Drop Test.)

3. Measure the "viscosity" of the Control and Test sample: The BB Drop Test
   a. Mix the yogurt so that it is a smooth uniform gel.
   b. Place a yogurt sample in the conical container, and inspect it to make sure there are no pockets of trapped air.
   c. Even off the top with a flat edged object (butter knife) so that the yogurt is at the same level as the top of the conical
   d. Prepare timer (can be on phone or stopwatch)
   e. Place BB at top of the yogurt and start timer as you release the BB (**Figure 9**).
   f. Document time that it take for BB to reach the bottom of the conical.
   g. Repeat for each yogurt sample. (2 measurements on Yogurt)
   h. Milk Samples: Measure the drop time for each milk only once.

(Notes: There are only two conicals. So, here is a working plan. Put Control milk in one and Control yogurt in the other. Drop a BB in the milk and measure the time to hit. For the two data points on the Control yogurt sample, drop one BB in, and measure the time to hit the bottom. Then drop another BB in the same conical and measure the travel time for it. NOW- clean both conicals, keep the BBs from going down the pipes, dry the conicals before starting on the Test milk and yogurt samples.)

4. The Lab Report Google Form will be available starting Monday, November 9, 2020. Email Ashley King arking2@go.olemiss.edu if you have a question.

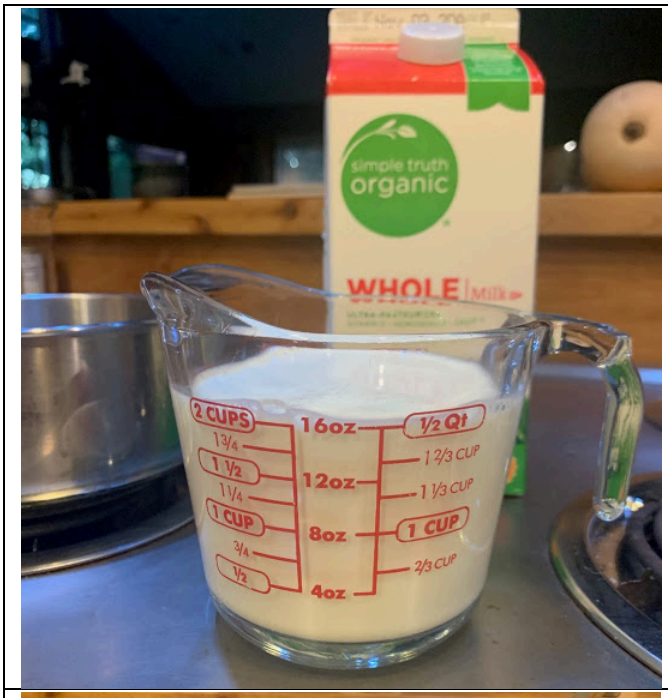	Figure 1: Measuring and Recording the volume, kind of milk and Brand of milk. Control milk = Whole Milk, Brand = Simple Truth Organic In this case, the Test milk (not shown) was 1% Milk, Brand = Simple Truth Organic. Samples (2 cups) were heated separately. First the Control, and then the Test.
	Figure 2: Heating the milk Milk is heated in a metal pot over a medium to medium high heat with constant stirring. Even a moment of negligence can cause the milk to burn to the bottom of the pot and you will have to start over.

	Figure 3: Boiling milk Since you are stirring constantly, it may be hard to discern whether the milk is actually boiling. When it starts to boil, if you are paying attention, you will notice the volume to increase- rapidly. It will overflow very quickly if you are not mindful.
	Figure 4: Preparing the Starter culture Take the yogurt container. Open the sealed lid and stir the contents until they are of uniform consistency. Get a Tablespoon, and overfill it with the yogurt. Then take the back of a knife and drag it across the spoon to ensure that you have exactly 1 Tbsp of culture added to the heated then cooled milk.
	Figure 5: Stir in the starter culture and then pour the inoculated milk into a glass container. Stir the hot milk until it cools to 100 decrees F. This is approximately body temperature. Add the 1 Tbsp of culture and stir very well to distribute it so that no clots of yogurt starter remain suspended in the milk. You want as uniform a solution as possible when starting the overnight growth phase. We used a large drinking glass as the

	container for the overnight culture growth.
	Figure 6: Label and Cover the culture Once the Control culture is stable in its incubating container, then you are ready to make the Test culture. (If you have 2 pots, you can start the heating when the first is cooling.) Regarding incubation overnight
	**Figure 7**: pH of Yogurt. Just sample the aqueous component of the yogurt and milk rather than submerging the paper in the opaque fluid/gel

Figure 8:
Key for assigning the value of the pH of yogurts and milks.

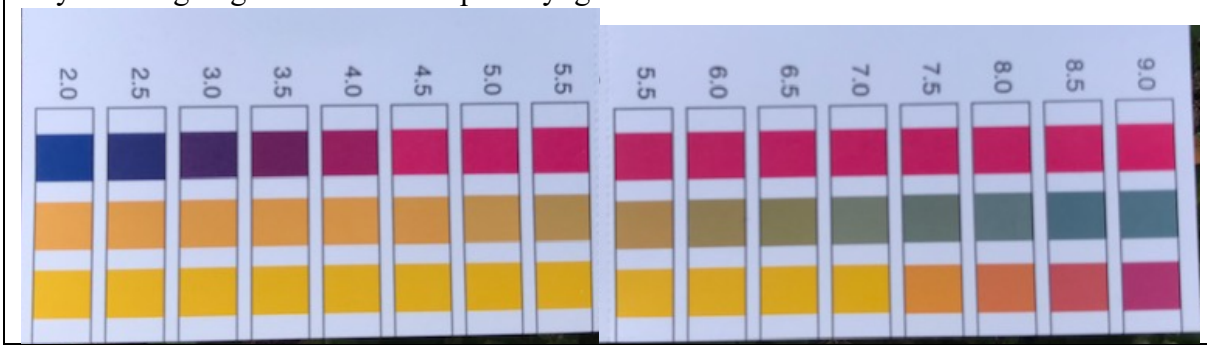

	**Figure 9:** The BB Test You have two 50 mL conicals. Fill one with milk and the other with yogurt. Record the time it takes for a BB to fall through the column of milk/yogurt to the bottom of the conical.

Nutrient label from Silk Brand Soy Milk.

Nutrition Facts
Valeur nutritive

Per 1 cup (250 mL) / par 1 tasse (250 mL)

Amount / Teneur	% Daily Value / % Valeur quotidienne
Calories / Calories 90 (380 kJ)	
Fat / Lipides 4.5 g	7 %
Saturated / saturés 0.5 g + Trans / trans 0 g	3 %
Cholesterol / Cholestérol 0 mg	0 %
Sodium / Sodium 90 mg	4 %
Potassium / Potassium 360 mg	10 %
Carbohydrate / Glucides 4 g	1 %
Fibre / Fibres 2 g	7 %
Sugars / Sucres 1 g	
Protein / Protéines 8 g	
Vitamin A / Vitamine A	10 %
Vitamin C / Vitamine C	0 %
Calcium / Calcium	30 %
Iron / Fer	8 %
Vitamin D / Vitamine D	45 %
Riboflavin / Riboflavine	25 %
Vitamin B_{12} / Vitamine B_{12}	50 %
Zinc / Zinc	10 %

We work hard to keep the information on this website up-to-date, but please check the label on your product for the most current nutrition facts.

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