Analysis of the Microbiome of Homebrewed Ginger Beer for Detection of Probiotics and Determination of Safety

by
Sushumna Sri Canakapalli

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Microbiology
(Honors Scholar)

Presented May 8, 2019
Commencement June 2019

AN ABSTRACT OF THE THESIS OF

Sushumna Sri Canakapalli for the degree of Honors Baccalaureate of Science in Microbiology presented on May 8, 2019. Title: Analysis of the Microbiome of Homebrewed Ginger Beer for Detection of Probiotics and Determination of Safety

.

Abstract approved:_____

Si Hong Park

Homebrewed ginger beer is a naturally fermented carbonated beverage that is flavored with ginger. Many people who make ginger beer at home, realized that it is a probiotic beverage which can aid in gastrointestinal health. The purpose of this thesis is to analyze the microbiome of homebrewed ginger beer in order to see if there is presence of any probiotic taxa of microbes as well as look at the general safety of its consumption. In this research ginger beer was made, measured for alcohol content, and performed microbiome analysis. The ginger beer had a very low alcohol content, similar to that of a non-alcoholic beer. The aerobic plate count of the ginger beer was as high as $1.62x10^6$ CFU/ml and the amount of yeast was as high as $3.58x10^6$ CFU/ml. The bacterial microbiome of the ginger beer did not contain any known probiotic OTUs, but did contain the genus Trabulsiella which could potentially act as a probiotic in humans. The microbiome did not contain anything that would make the ginger beer seem unsafe for human consumption.

Key Words: Fermented Beverages, Microbiology, Probiotics

Corresponding e-mail address: canakaps@oregonstate.edu

Analysis of the Microbiome of Homebrewed Ginger Beer for Detection of Probiotics and Determination of Safety

by
Sushumna Sri Canakapalli

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Microbiology
(Honors Scholar)

Presented May 8, 2019
Commencement June 2019

Honors Baccalaureate of Science in Microbiology project of Sushumna Sri Canakapalli presented on May 8, 2019.

APPROVED:

_____

Si Hong Park, Mentor, representing Food Science and Technology

_____

Christopher Curtin, Committee Member, representing Food Science and Technology

_____

Thomas Shellhammer, Committee Member, representing Food Science and Technology

_____

Toni Doolen, Dean, Oregon State University Honors College

I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

_____

Sushumna Sri Canakapalli, Author

Acknowledgements

I would first like to thank my mentor, Dr. Si Hong Park for saying yes to my crazy ginger beer fueled ideas and making this entire thesis possible. I would also like to thank Sang In Lee and Jungmin Choi for helping me learn the technical aspects needed to accomplish my research.

Next I would like to thank Tadvana Canakapalli, whose spectacular accomplishments were the catalyst for completing this thesis.

Finally I would like to thank the members of the Corner House, who were the greatest source of support and commiseration that a college undergraduate could ever ask for.

Table of Contents

List of Tables and Figures

Introduction

Ginger beer is a carbonated beverage flavored with ginger. Homebrewed ginger beer is made with a starter culture called the "ginger bug", named from the ginger used to make it and the colloquial term for microbes, "bugs". A ginger bug is made through a natural (or wild) fermentation process, and is often made to be non-alcoholic. A wild fermentation is one that requires no input of yeast, as it uses the yeast naturally found on the substrate being fermented. Ginger beer can have varying levels of sweetness, depending on the amount of sugar added, as well as the amount of sugar consumed during fermentation. While ginger beer can have a wide range of flavors based on ingredients used and microbes present, it can be recognized by the distinct spiciness provided by the gingerroot.

Zingiber officinale, or ginger, is a plant rhizome that is used across the world as a spice in foods and beverages. It can be found in tropical and subtropical regions and is often grown in nutrient rich soil with ample water supply (1). Ginger is popular, not only for its unique flavor, but for its medicinal properties that help aid ailments such as nausea, cold symptoms, arthritis, joint and muscle pain, diarrhea, and motion sickness (2). Ginger is also known to have antimicrobial and antifungal properties (1).

To participate in a natural fermentation, there must be a presence of microbes on the substrate being fermented. Past studies looking at the microbial composition of ginger, have reported bacterial counts as high as 10^{10} CFU/g and yeast and mold counts as high as 10^{7} CFU/g on fresh ginger rhizomes (3). Ginger-based beverages similar to ginger beer that have been fortified with probiotic bacteria have been found to have between $10^{2.25}$ and $10^{9.11}$ CFU/ml of the bacteria artificially added (3).

Homebrewed ginger beer, in comparison to commercially available soft drinks, not only differs in flavor but in that it contains live microbes from the fermentation process. Many people who make their own ginger beer claim that it is a probiotic food because of the live culture. While numerous fermented foods have health benefits due to what fermentative microbes produce, such as various B vitamins and fatty acids, this does not necessarily mean that they are probiotic (4). There is no support to the claims of ginger beer being probiotic or healthy if its microbial content is not assessed.

Probiotics are "live microorganisms which when consumed in adequate amounts, confer a health effect on the host" (5). These health effects include helping the host digest food and preventing pathogenic microbes from harming the host. Consultants of the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) have certain guidelines by which they suggest classifying organisms as probiotics. To be a probiotic, an organism must be able to proliferate in the gut of the host without being damaged by gastric juices or being digested (6). It is also important to know the specific strain of the organism, as health effects can vary past the species level (6). Some ways to perform strain typing include pulsed-field gel electrophoresis, randomly amplified polymorphic DNA, and determination of presence of extrachromosomal genetic elements (7). Many known probiotic bacteria can be found in the can be found in the genera *Lactobacillus* and *Bifidobacterium* (6). For example, *Bifidobacterium infantis* and *Lactobacillus acidophilus* have been found to have probiotic effects in infants (8). Neonates given these bacteria saw a 60% reduction in necrotizing enterocolitis compared to neonates not treated the previous year (8).

Next generation sequencing can be used to find out what types of organisms are present in an environment. Illumina MiSeq is a next generation sequencing instrument that uses

sequencing by synthesis. MiSeq has four main steps including sample prep, cluster generation, sequencing, and data analysis (9). Sample preparation adds adaptors to each of the DNA fragments. It also adds the sequencing binding site, indices, and complementary regions (9). Cluster generation occurs through a process called bridge amplification, which ultimately connects every fragment to a flow cell in clusters (9). The fragments are then sequenced by synthesis. Fluorescent nucleotides are base paired with the fragments in each cluster, which determines both the bases in each cluster and the length of each fragment (9). These "reads" are then recorded. The reads are clustered together based on similarity and compared to a reference genome to identify what the DNA is (9).

Materials and Methods

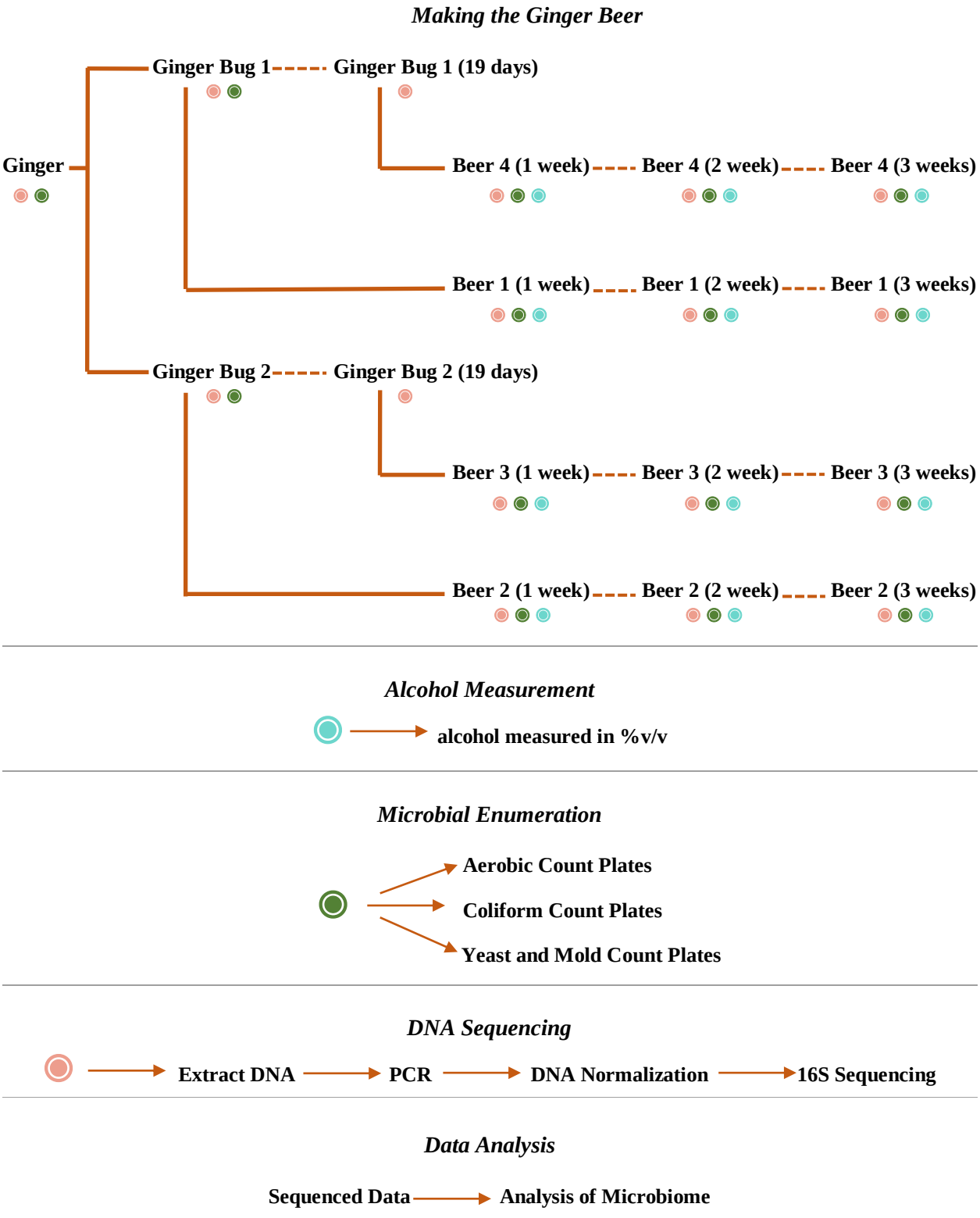

Figure 1. Methods Overview

1. Making the Ginger Beer

Two replicates of ginger beer were made based on Recipe 1 (see Appendix A). Two ginger bug replicates were made by combining ginger, sugar and water. Once ready (see Figure 2), each was used to make a batch of ginger beer (replicates one and two). Upon opening the ginger beer fermenting for one week, very little effervescence was present. This prompted a third and fourth replicate of ginger beer to be made using Recipe 2 (see Appendix A). Both replicates three and four were made using the ginger bug left over from replicates two and one respectively. Since the microbial composition of a live culture can change over time, DNA was extracted again from the two replicates of ginger bug on the day that the third and fourth ginger beer replicates were made (19 days since the first two ginger beer replicates were made and the first time DNA was extracted from the ginger bugs).

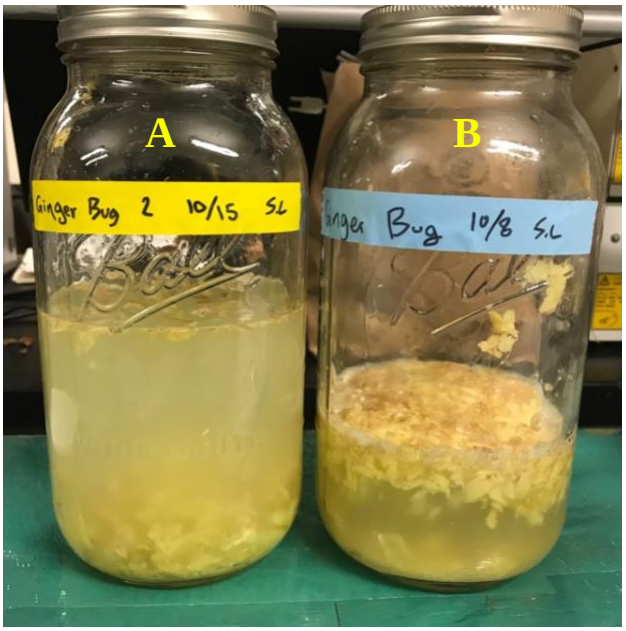

Figure 2. A: Ginger bug after it was just made. No formation of bubbles or presence of effervescence. B: Completed ginger bug that was already used to make ginger beer. The grated ginger has all risen to the top underneath a layer of foam.

Figure 3. Replicate four of ginger beer bottled and sealed.

2. Microbial Enumeration

Microbes were enumerated using the 3M™ Petrifilm™ Aerobic Count Plates (AC) (3M, MN, USA), Coliform Count Plates (3M MN, USA), and Yeast and Mold Count Plates (3M, MN, USA) for every step of the ginger beer making process: raw gingerroot; completion of ginger bug; and ginger beer fermented for one, two, and three weeks. Two technical replicates for every dilution were made according to the dilution scheme shown in Figure 4. The results of the microbial counts are shown in Figures 4, 5, and 6.

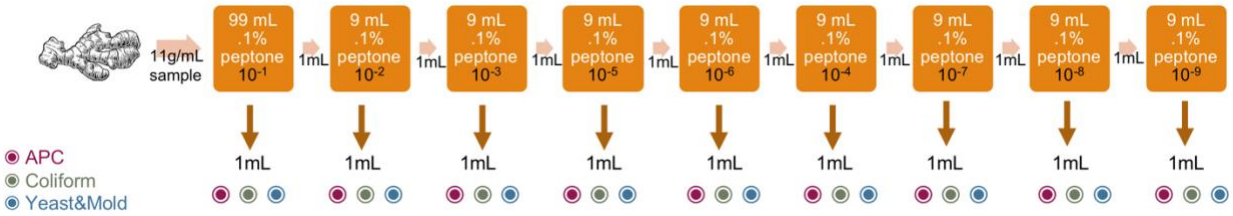

Figure 4. Dilution scheme of raw gingerroot, ginger bug, and ginger beer. Samples of ginger were measured to 11 grams while samples of ginger bug and ginger beer were measured to 11 milliliters.

3. Alcohol Content

Alcohol content was measured using an Anton-Parr DMA™ 4500 M (Anton Paar GmbH, Graz, Austria) and Alcolyzer ME (Anton Paar GmbH, Graz, Austria) for the completed ginger beer fermented for: one, two, and three weeks. A water check was performed on the instruments using deionized water to validate results. The samples of ginger beer were filtered to remove any solid matter and then placed in polystyrene vials in duplicate. The alcohol content was recorded for each vial and averaged for every sample.

4. DNA Sequencing

DNA was extracted for every step of the ginger beer making process. Extraction was done using the Qiagen DNeasy PowerFood Microbial Kit. Amount of DNA extracted was measured using the Invitrogen Qubit 4 fluorometer with the Qubit dsDNA BR assay kit. DNA extractions were diluted to have a concentration of 10 ng/µl for use in PCR. For samples below 10 ng/µl, the amount of DNA used in PCR reaction was increased to get as close to 10 ng without exceeding 6 µl per sample. PCR was performed for all samples to amplify the 16S DNA. Success of amplification was determined by gel electrophoresis. DNA concentrations were standardized using Invitrogen SequalPrep Normalization Plate (96) Kit. DNA concentration was measured again for several samples using the Qubit dsDNA BR assay kit. All samples were sequenced at the 16S region.

5. Data Analysis

Sequences were analyzed using Qiime2. Qiime2 was activated through Terminal, and the "Moving Pictures" tutorial was used as a basis to analyze the data (10). Sequences were demultiplexed and a summary was generated. The demultiplexed sequences were then corrected using the DADA2 pipeline to help remove chimeric sequences. The corrected sequences were

used to produce various metrics including alpha rarefaction and Bray-Curtis beta diversity. Taxonomic data was produced in order to see the variety of taxa within the microbiome.

Results/Discussion

Colony Counts on Petrifilms™

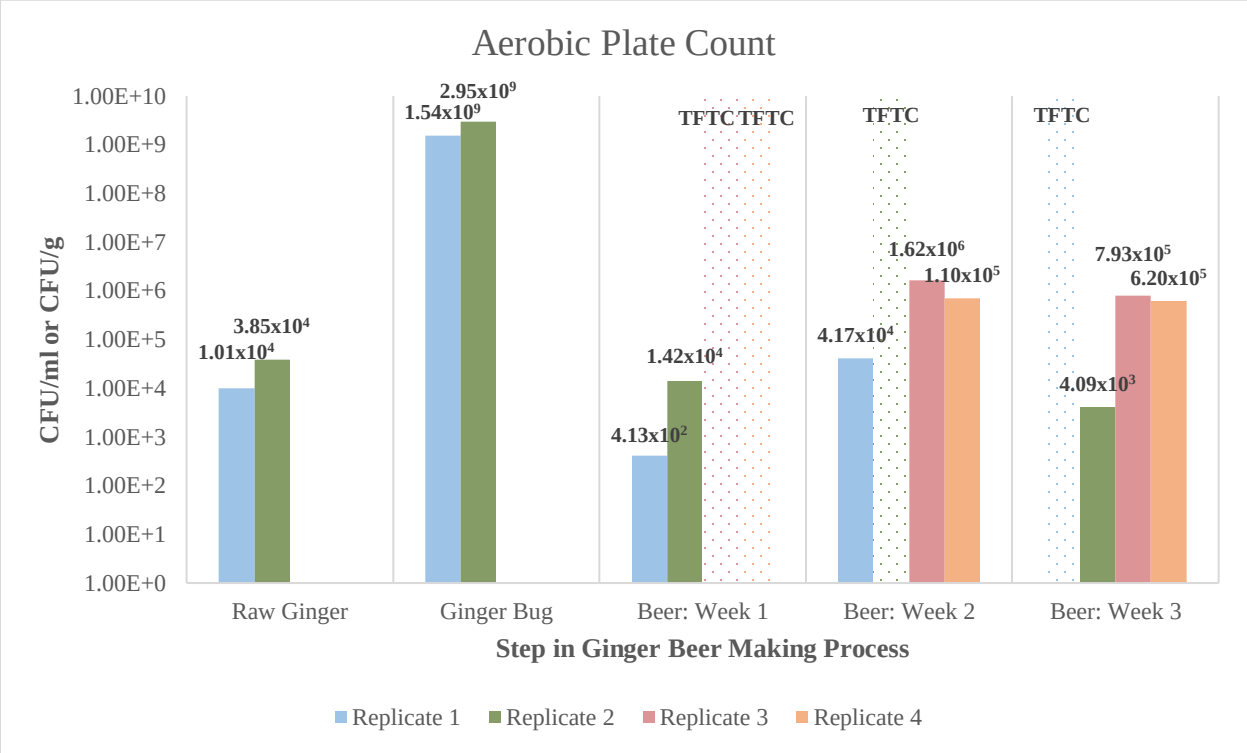

Figure 5. Average aerobic plate counts for every sample of ginger, ginger bug, and ginger beer using 3M™ Petrifilm™ Aerobic Count Plates. Averages were taken from every Petrifilm™ with a statistically significant count (between 25 and 250 colonies). Samples that had no colony counts above 25 were considered "too few to count" and are denoted as TFTC in the figure. Samples that had no colony counts under 250 were considered "too numerous to count" and are denoted as TNTC in the figure. Colony counts for ginger samples were measured in colony forming units per gram. All other samples were measured in colony forming units per milliliter.

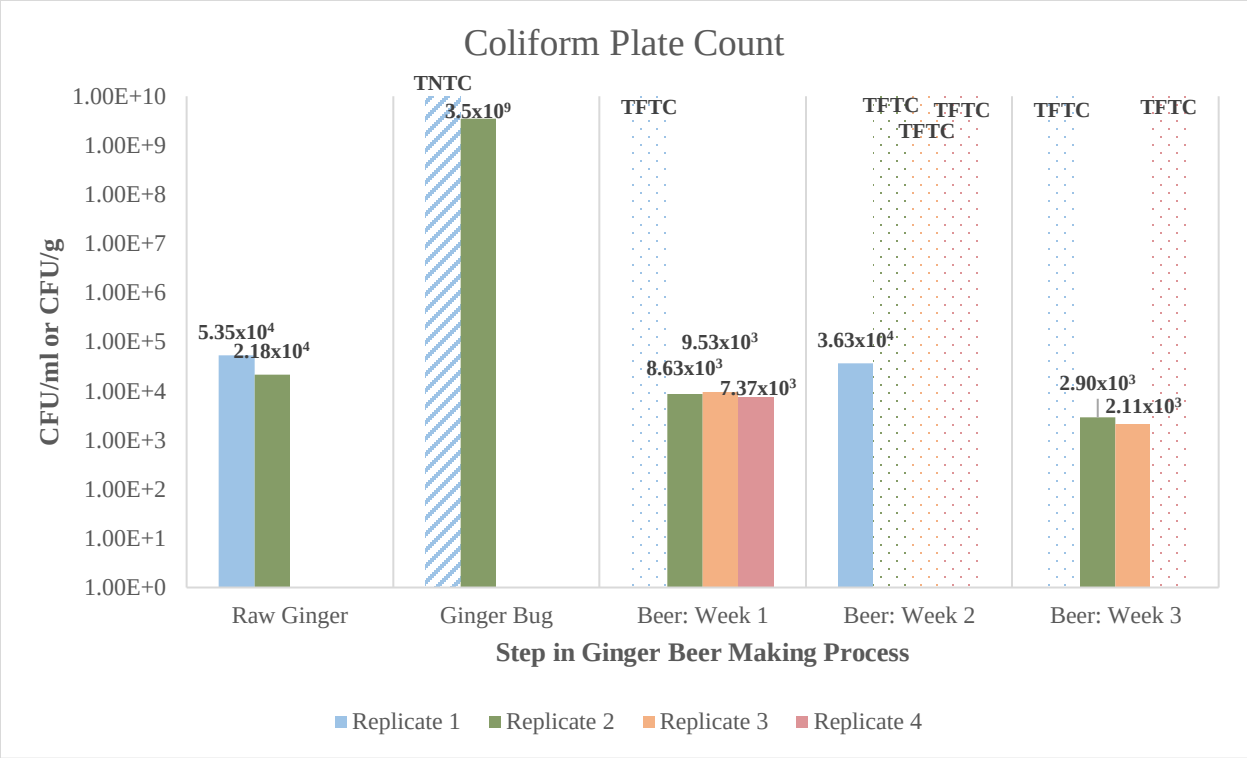

Figure 6. Average coliform counts for every sample of ginger, ginger bug, and ginger beer using 3M™ Petrifilm™ Coliform Plates. Averages were taken from every Petrifilm™ with a statistically significant count (between 25 and 250 colonies). Samples that had no colony counts above 25 were considered "too few to count" and are denoted as TFTC in the figure. Samples that had no colony counts under 250 were considered "too numerous to count" and are denoted as TNTC in the figure. Colony counts for ginger samples were measured in colony forming units per gram. All other samples were measured in colony forming units per milliliter.

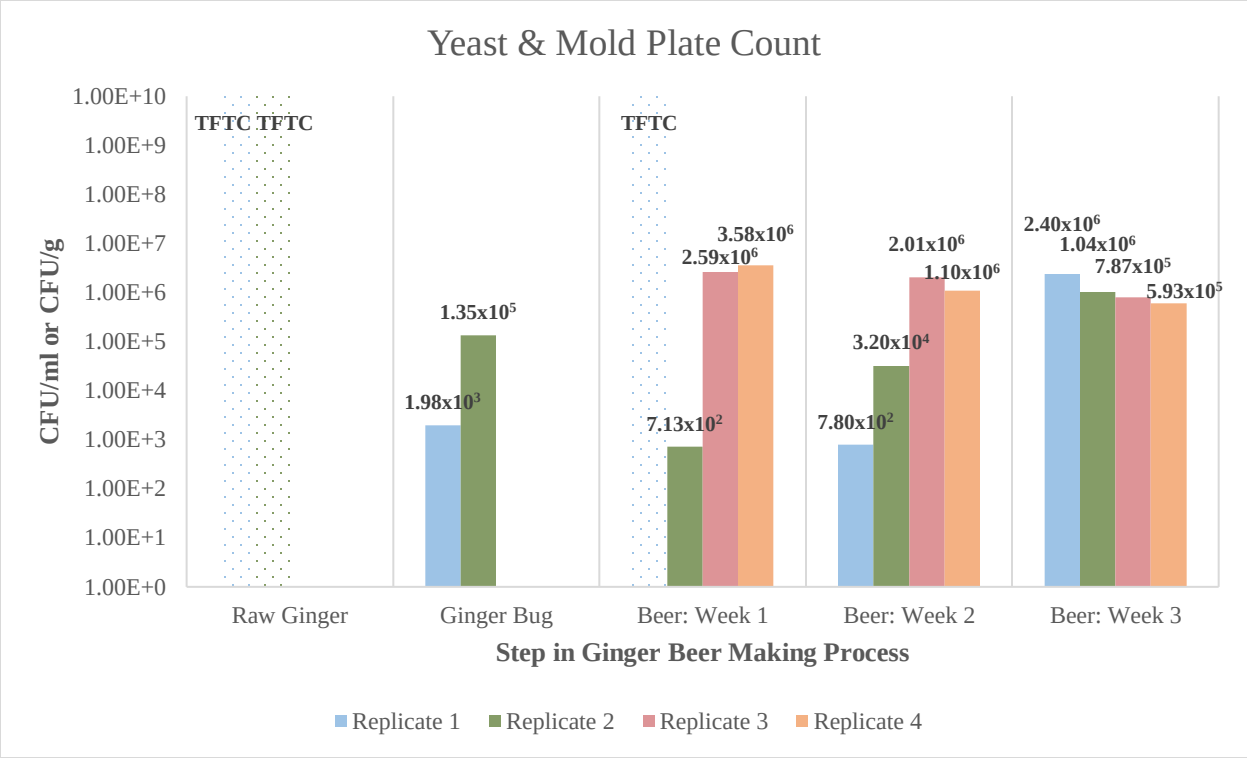

Figure 7. Average yeast counts for every sample of ginger, ginger bug, and ginger beer using 3M™ Petrifilm™ Yeast and Mold Plates. Averages were taken from every Petrifilm™ with a statistically significant count (between 25 and 250 colonies). Samples that had no colony counts above 25 were considered "too few to count" and are denoted as TFTC in the figure. Samples that had no colony counts under 250 were considered "too numerous to count" and are denoted as TNTC in the figure. Colony counts for ginger samples were measured in colony forming units per gram. All other samples were measured in colony forming units per milliliter.

The number of colonies counted on the 3M™ Petrifilm™ Aerobic Count Plates for raw ginger were within 10^4 CFU/g. This is well under the maximum expected colony count for gingerroot based on other studies (2). The ginger bugs had the highest aerobic counts, which makes sense since they act as the starter culture for the ginger beer. The ginger beer itself had aerobic counts ranging from "too few to count" (under 25 colonies per plate) to 10^6 CFU/ml. The difference in amount of bacteria between samples does not seem to correspond to how long the beer had been fermenting for. It also does not seem to relate to how much ginger bug was added to make each sample. Replicates 1 and 2 and replicates 3 and 4 had the same amount of ginger bug added and there is still a large range between those replicates in colony counts.

The number of colony counted on the coliform Petrifilms™ for the ginger beer were relatively low, mostly being "too few to count", not exceeding 10^4 CFU/ml. Having a low amount of coliform bacteria in foods and beverages is considered a positive outcome, since coliforms can be used to indicate potential fecal contamination as well as the presence of *E. coli*. Since only a small portion of all coliforms act as human pathogens, the chance that the small amount of coliform bacteria in the ginger beer sample are dangerous and originate from fecal contamination is low.

The number of yeast colonies counted on the yeast and mold Petrifilms™ for the ginger beer ranged from "too few to count" up to 10^6 CFU/m, most samples being closer to the latter. There were no mold colonies detected. Since there are no other ginger beer data to compare these counts to, the amount of yeast counted is assumed to be normal. The DNA of the yeast was not sequenced, so there is not enough information to state what type of yeast is present in these samples. However the relatively consistent presence of yeast indicates that they probably played a role in the fermentation process, as is common with many fermented foods and beverages.

Variation and Statistical Significance amongst Colony Counts

Table 1. Anova Test and Standard Deviation for APC films

APC						
Anova: Single Factor-						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	8.3972×10^{18}	4	2.0993×10^{18}	14.7829657	0.00159333	4.12031173
Within Groups	9.9405×10^{17}	7	1.4201×10^{17}			
Total	9.3912×10^{18}	11				
Standard Deviation-						
9.24×10^{8} CFU/mL						

Table 2. Anova Test and Standard Deviation for Coliform films

Coliform						
Anova: Single Factor-						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1.0889×10^{19}	4	2.7222×10^{18}	2.1557×10^{10}	6.4556×10^{-21}	6.38823291
Within Groups	5.0511×10^{8}	4	1.26×10^{8}			
Total	1.0889×10^{19}	8				
Standard Deviation-						
1.17×10^{9} CFU/mL						

Table 3. Anova Test and Standard Deviation for Yeast and Mold films

Yeast and Mold						
Anova: Single Factor-						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	5.3142×10^{12}	4	1.3286×10^{12}	0.91410252	0.50025922	3.83785335
Within Groups	1.1627×10^{13}	8	1.4534×10^{12}			
Total	1.6941×10^{13}	12				
Standard Deviation-						
1.19×10^{6} CFU/mL						

ANOVA tests were performed between groups (raw ginger; ginger bug; and fermented ginger beer fermented at one, two, and three weeks) for colony counts of Aerobic Count, Coliform, and Yeast and Mold Petrifilms™ and their standard deviations were calculated (see Tables 1, 2, and 3). The Yeast and Mold Petrifilms™ had quite a high p-value, suggesting little variation between groups. One possibility for this is because there were no viable counts for the raw ginger. Since there were no statistically significant plates (plates with over 25 colonies) in this group, none of them could be counted. There should likely be a large difference between the raw ginger and the other groups due to the lack of fermentation in the raw ginger, but this cannot be conformed due to the lack of viable data in the raw ginger group. Another possible reason for

the high p-value is due to the similarity between the ginger bug group and all the ginger beer groups. The standard deviation amongst all the samples for the Yeast and Mold films is also lower than the ones for APC and Coliform. This, along with the ANOVA results suggest that the amount of yeast present does not change significantly between stages of the ginger beer making process, nor due to time fermented. Statistically significant colony counts of the raw ginger would need to be attained to get a more accurate picture of the variance between all groups. The variation between groups for the APC and Coliform films are significant. While the source of variability cannot be identified through the ANOVA test, the raw counts allude to the variability coming from the ginger bug group. The colony counts are much higher than any other stage in the ginger beer making process. This would make sense, since the fermentation of the ginger bug creates a high concentration of bacteria.

Alcohol Content of Ginger Beer

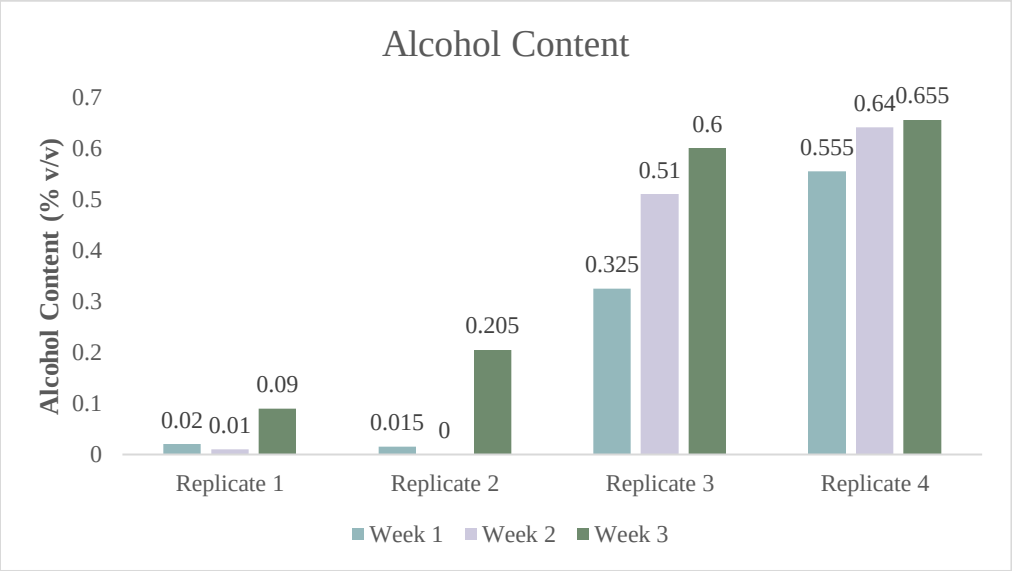

Figure 8. Alcohol Content of Ginger Beer

The alcohol content in the ginger beer ranged from 0 to .655% by volume (see Figure 8), which is not particularity high (for reference, a nonalcoholic beer is around .5% by volume). This was expected, as homebrewed ginger beer is not usually made to be an alcoholic beverage. For the third and fourth replicate of ginger beer, there was at least .3% alcohol by volume in one week (see Figure 8). For the first and second replicate, it took fermenting for three weeks to even have more than a negligible amount of alcohol. The presence of alcohol seemed to correspond with the amount of effervescence the ginger beer had, meaning the higher the alcohol content, the more fizz present when the ginger beer sample was opened. This could be an indicator that there is some relation between ethanol fermentation and CO_2 fermentation. What is more likely is that the third and fourth replicates had a higher alcohol content than replicates one and two because of how aged the ginger bugs were by the time they were made. Since it had been 19 days since anything had been added or modified in the ginger bugs, it was a chance for the ginger bugs to ferment further. When they were opened after those 19 days, they had a notable alcoholic scent. This might suggest that replicates three and four did not produce alcohol faster than the first two replicates, but rather had alcohol directly added to them via the ginger bugs.

Microbiome

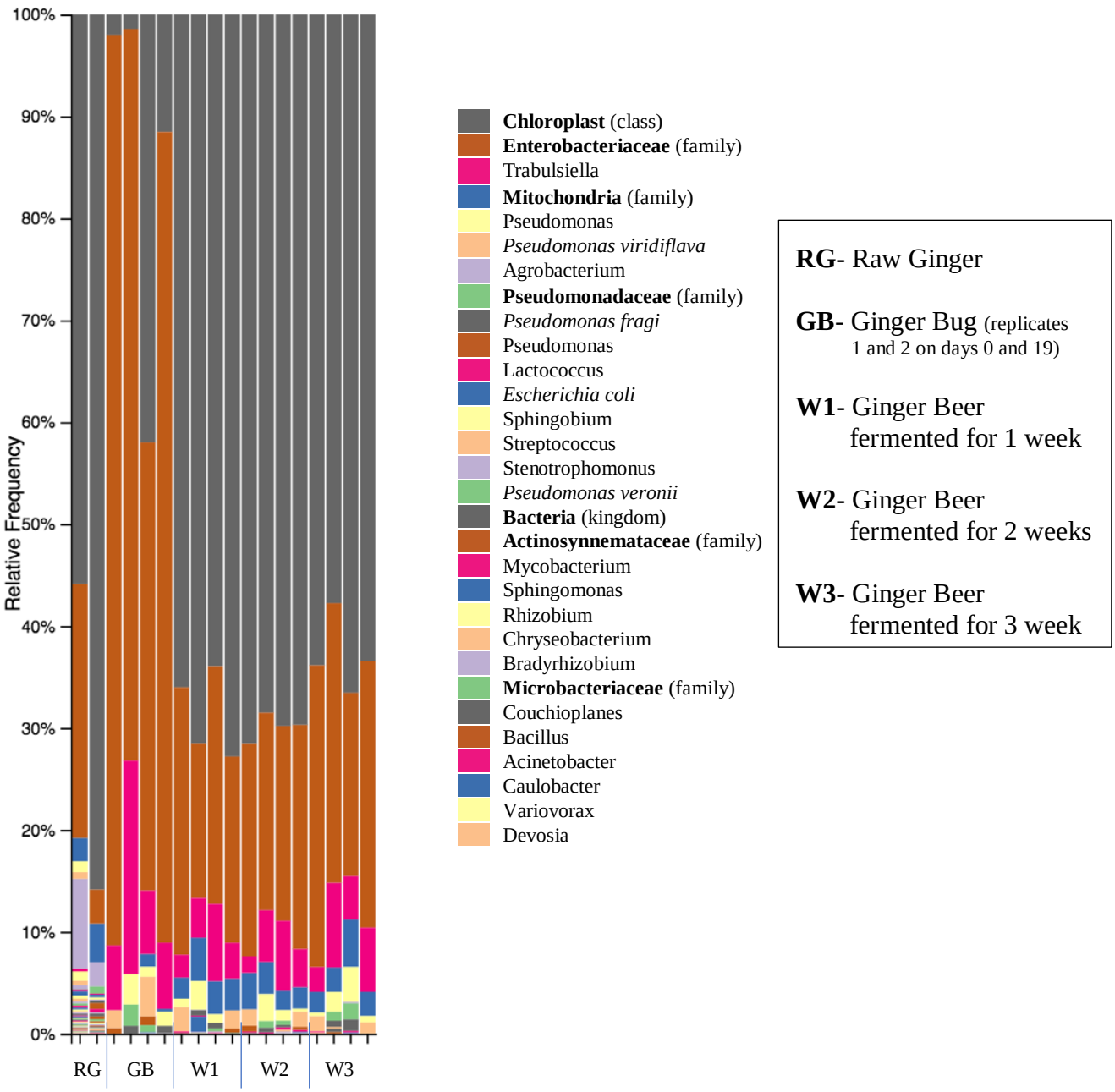

Figure 9. Taxa Plot- First 30 most abundant taxa listed by genus and species. If species is not known, just the genus is listed. If neither is known, the highest taxonomic level is listed in parenthesis.

Alpha Diversity

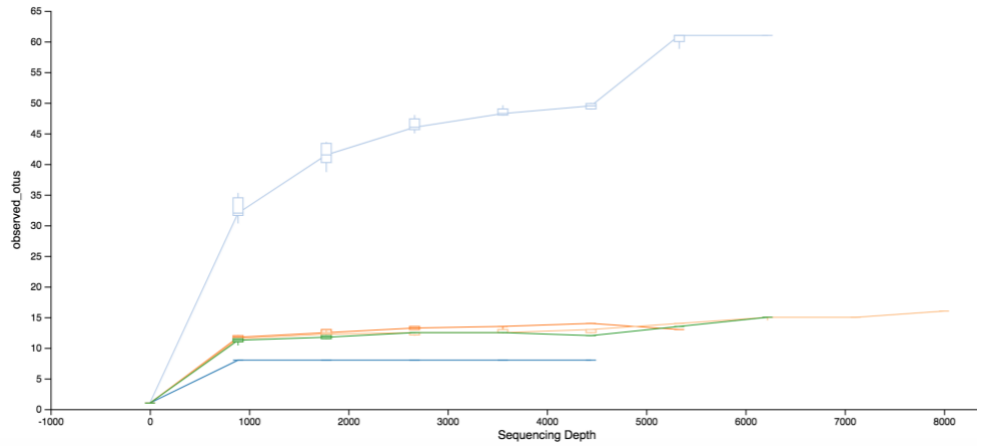

Figure 10. Alpha Diversity- Alpha Rarefaction of Observed OTUs

The alpha diversity is quite consistent amongst all of the groups besides the raw ginger (see Figure 10). The high and varying number of Operational Taxonomic Units (OTUs) within the raw ginger group is likely due to the amount of handling the raw ginger received. It passes through many hands from farm to grocery store and came in contact with many surfaces. Because the bacteria is coming from various environments, it is not expected for there to be much consistency between samples of ginger. One sample could have easily come in contact with many bacteria that the other samples did not. The alpha diversity of the other groups is far less, because the fermentation process streamlines the types of bacteria present. The OTUs in these groups will only be able to include the bacteria that can perform the fermentation, and those that can survive presence of the fermentation products produced. Since this significantly drops the number of possible bacteria each sample can contain, the samples will not only have a smaller OTU counts, but also be much more similar to each other. Many bacteria that would

have been a cause for variation between samples, die out, not being able to survive the fermentation conditions.

Beta Diversity

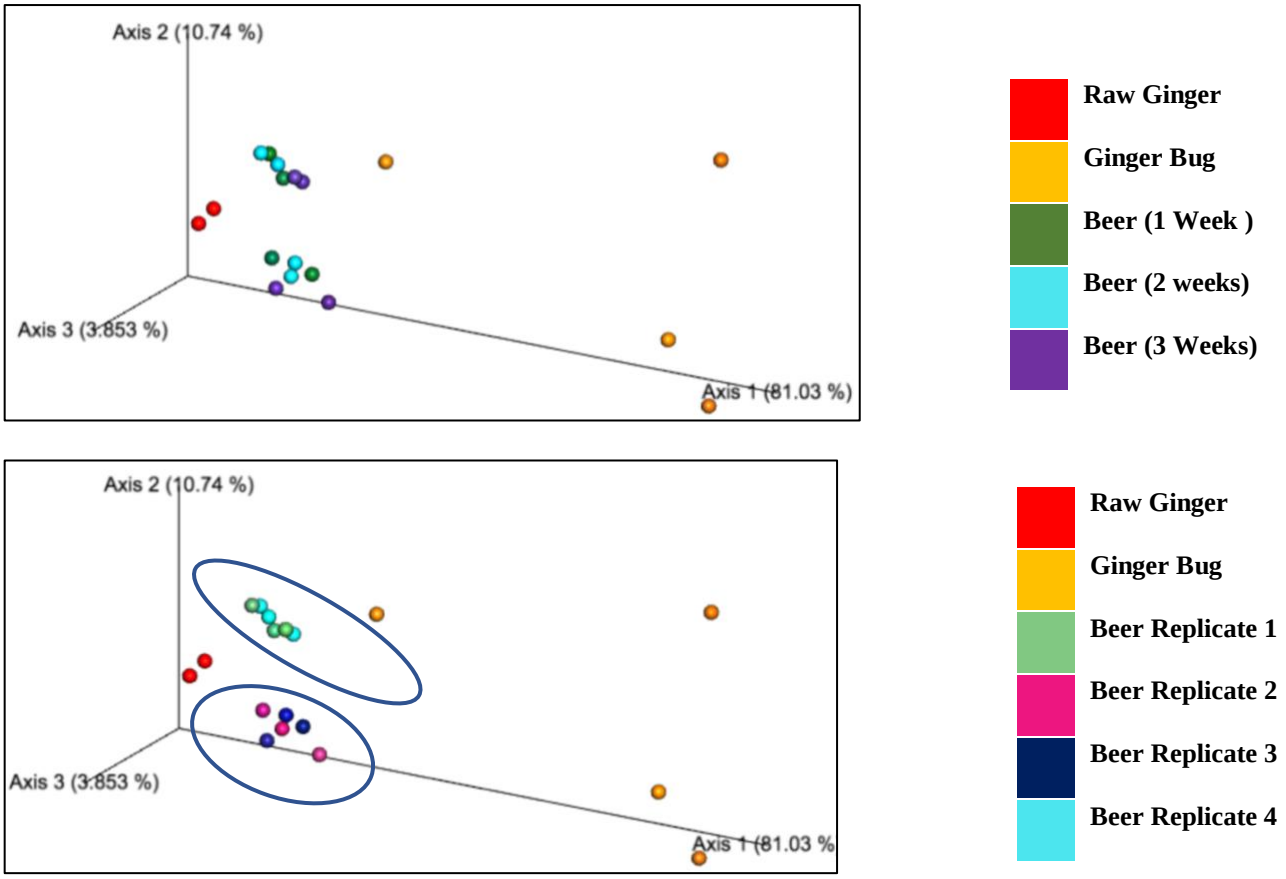

Figure 11. Beta Diversity- Bray Curtis Model

The Bray-Curtis model (see Figure 11) shows some clustering between stages of the ginger beer making process. The two samples of raw ginger are more similar to each other than they are to the ginger bugs they each were added to. The ginger bugs are the most different from the other samples and spread farther from the other samples as well as each other. This is interesting because the raw ginger has a larger relative proportion of each succeeding taxa after

the first two most abundant taxa (see Figure 9). That would make it seem like raw ginger should be the most different from the other samples. The diversity of the ginger bugs might be due to the large amount of Enterobacteriaceae that they have. Three of the four ginger bug samples have more than twice the relative abundance of Enterobacteriaceae than any of the other samples. This is probably what is causing them to have a distance from the other samples. Though the first ginger bug sample taken on 19 days does not cluster with the other types of samples, it does not cluster as closely with the other ginger bug samples. This may be due to the smaller percentage of Enterobacteriaceae that it has compared to the other ginger bug samples.

While there are clear distinctions in beta diversity between raw ginger, ginger bugs, and ginger beer, there is not much difference between ginger beer fermented for one, two, and three weeks. There is however a distinction between the different replicates of ginger beer. Replicates one and four cluster together, while replicates two and three cluster together. This is based on the original source of the inoculum, as replicates one and four both came from the first ginger bug, while two and three came from the second ginger bug.

Chloroplast

Large frequencies of the taxa in the raw ginger are classified as Chloroplasts. These most likely are coming from cells of the ginger itself and drop drastically in relative abundance in the ginger bug (11). The relative abundance increases again in the ginger beer. This is probably do to the incorporation of raw ginger juice, adding more chloroplast to the microbiome. This may also be a reason that the raw ginger and the ginger beer are not as diverse from each other as the ginger bug. Sequencing of chloroplasts is difficult to avoid due to their DNA's similarity to bacterial DNA and the amount of plant cells present in the ginger, but it is recommend that they be removed to avoid contamination of the data set (11).

The large amount Chloroplast DNA may have something to do with the time it took to ferment the ginger beer. Since the additional ingredients were not boiled (the water and the ginger juice), any chloroplast cells present in those ingredient were not killed. The amount of outside cells entering the mixture might have inhibited the fermentation, explaining why it took three weeks for some of the replicates to show a presence of effervescence or alcohol.

Enterobacteriaceae

Bacteria in the family Enterobacteriaceae, were present in high relative abundance in all samples, especially those of the ginger bug samples. While Enterobacteriaceae gets its name from having enteric bacteria within the family, this is not true for every genus or species (12). Nonetheless, Enterobacteriaceae is often used as a standard for hygiene by food manufacturers (13). Large presence of Enterobacteriaceae in the samples may be an indicator of poor hygiene in the location where the ginger beer was being made. However genus level information would be needed to claim any roles the Enterobacteriaceae might be playing. *E. coli* is present at less than 2% of the community of the samples it is in. The secondary fermentation process may have had an effect on the abundance of the Enterobacteriaceae since the ginger beer has a lower relative abundance of Enterobacteriaceae than the ginger bugs. It is more likely that the addition of raw ginger juice increased the chloroplast content of the beer, making it seem like the Enterobacteriaceae content declined. Fermentation processes in the beer could have shifted the community as well. Many species in the Enterobacteriaceae family can perform carbon dioxide and ethanol fermentation, making them possible contributors in the ginger beer's final composition.

Home Brewed Ginger Beer as a Probiotic

The genus Lactococcus was present in almost every ginger beer sample. However, it was found at low relative abundancies and was present in the raw ginger as well. This means that even if the species of Lactococcus present are probiotic, they would play a small role in the ginger beer due to their minimal quantity (no more than .3% of any sample's community). The Lactococcus was also equally as present in the raw ginger as it was in the ginger beer, which means that the fermentation process did not cause it to proliferate. The same amount of Lactococcus would be consumed from eating the ginger as drinking the fermented ginger beer.

Aside from the well-known genera that contain probiotic species (Lactococcus, Lactobacillus, and Bifidobacterium), other taxa of bacteria have the capability to be probiotic as long as they are able to "confer a health effect on the host". One such potential genus that proliferated in the ginger beer was Trabulsiella. Trabulsiella is a genus of Gram-negative rod bacteria. One of the known functions of *Trabulsiella* sp. is helping termites break down lignin during the digestion of wood (14). Due to this fermentative breakdown, isolates of *Trabulsiella* sp. from termite guts have been looked at as candidates for probiotics in humans to help break down cellulose during digestion (15). Since there are very few known species of Trabulsiella, it is likely that the Trabulsiella within the ginger beer has the ability aid in digestion of cellulose.

Probiotic yeast could also be present in the ginger beer, but additional analysis would need to be done to figure this out. Even if large amounts of known probiotics species were found in the beer, more testing would need to be done to see if ginger beer consumption would be associated with positive health effects.

Safety Concerns of Consuming Home Brewed Ginger Beer

Apart from the other Enterobacteriaceae, one sample of ginger beer (replicate two at one week) contained a relatively large proportion of *Escherichia coli* at 1.48% of the total bacterial composition. As the ginger beer was made in an environment that comes in contact with many microbes, it is most likely that the ginger beer sample was exposed to a harmless lab strain of *E. coli.* Since the other ginger beer samples did not contain *E. coli*, the presence of it was most likely a chance contamination that occurred when bottling that specific sample. While it is good that the growth of *E. coli* was not common across the beer (not a normal community member for the fermentation), it does call into question of the ease of contamination of a homebrewed beverage. If ginger beer brewed in a controlled lab setting was able to be contaminated, then it would be just as easy to get contamination in a home kitchen that or may not be more sanitary.

There was no presence of certainly pathogenic bacteria in any of the samples of ginger, ginger bug, or ginger beer. This, along with the low coliform counts within the beer itself, makes the ginger beer made in all four replicates safe for consumption from a microbial perspective.

In the Future

There are multiple additional experiments that could be conducted to make the research done more complete. The first would be to sequence the eukaryotic DNA is order to find out what kind of yeast is present in the ginger beer. Since yeast can play a large role in food fermentations, it would have been beneficial to see the specific types of yeast present.

Another step that could be added, is to perform the fermentations in multiple different locations, especially the fermentation of the ginger bugs. Since a natural fermentation uses no outside input of yeast or bacteria, the variations of microbes in the environment could have a large impact on the final ginger beer microbiome. By fermenting the ginger bugs in multiples

locations, the consistency of microbes could be assessed, and better conclusions could be made about whether just the ginger beer made in the lab was safe and/or probiotic, or if ginger beer made in a variety of individuals' kitchens would have similar microbiomes.

Starting with a good ginger beer recipe from the start would have improved the testing that was performed. While ginger beer was successfully made and data was collected, the recipe went through some trial and error (see Appendix A). Because the first ginger beer made did not have a noticeable amount of effervescence after one week of fermenting, the recipe was adjusted. Thinking the problem was not using a large enough amount of the ginger bug (since the ginger bug was very effervescent), the second recipe used, had a large proportion of ginger bug in it. This did make some difference in the effervesce after one week of fermentation, but still not much. It did however lead to a very fizzy beverage by three weeks of fermentation. So while a successful ginger beer was eventually made, the process could have been restructured to reach a better end product faster and with only one recipe.

Conclusions

Difference in Beer based on Replicate and Time Fermented

There is a difference in taxa that is noticeable between replicates of ginger beer. The difference is based off of which ginger bug the replicate was made from. While the amount of alcohol and effervescence present also depended on the replicate, these factor changed with time as well, unlike taxa which did not adjust very much as the beer fermented for longer. The total amount of bacteria and yeast in each sample was not particularly different between replicates, though there was some significance in the difference with time for bacteria.

Based on the bacterial DNA that was sequenced, there is minimal evidence to support that the ginger beer made could be considered probiotic. There were no OTUs present that are traditionally considered beneficial for human health. There were bacteria that have the potential to have probiotic properties, but further testing would need to be done to confirm this.

The ginger beer made should be safe for human consumption from a microbial standpoint. While there were some coliforms present in the beer, the amount was minimal and included only one incident of *E. coli*, most likely a harmless laboratory strain.

For those who choose to naturally ferment ginger beer in their homes, they can be relatively confidant that the final beverage is safe to drink (given that environment that they make it in is relatively sanitary), but should be aware that drinking the ginger beer would most likely have little or no probiotic effects.

References

1. Mendi S, Nain C, Imele H, Ngoko Z, Carl MF. 2009. Microflora of fresh ginger rhizomes and ginger powder produced in the North-West Region of Cameroon. J Anim Plant Sci. 4: 251-260.
2. Herbs at A Glance. National Center for Complementary and alternative medicine. Ginger. 2008. U.S department of health and human services. National institute of health.
3. Hezekiah Adeniran A, Terkula Ukeyima M, Henrietta Abiose S. 2010. Microbiological assessment of probioticated ginger-based beverages. Nutr Food Sci. 40:209–220.
4. Katz S. 2003. Wild fermentation: the flavor, nutrition, and craft of live-culture foods. Chapter 1: The health benefits of fermented foods. p5-12. Chelsea Green Publishing Company. Vermont.
5. Morelli L, Capurso L. 2012. FAO/WHO Guidelines on Probiotics: 10 Years Later. J Clin Gastroenterol. 46: S1-S2.
6. Probiotics in food: Health and nutritional properties and guidelines for evaluation. FAO food and nutrition paper 85. FAO and WHO. 2006. ISSN 0254-4725
7. Reid G, Jass J, Sebulsky T, McCormick J. 2003. Potential Uses of Probiotics in Clinical Practice. Clin Microbiol Rev. 16: 658-672.
8. Hoyos, A. B. 1999. Reduced incidence of necrotizing enterocolitis associated with enteral administration of Lactobacillus acidophilus and Bifidobacterium infantis to neonates in an intensive care unit. Int. J. Infect. Dis. 3:197–202.
9. Illumina. 2016. Illumina Sequencing by synthesis. Accessed April 15, 2019. https://www.youtube.com/watch?v=fCd6B5HRaZ8

10. Qiime2docs. 2016. “Moving Pictures” tutorial. Version: 2018.11. Qiime 2. https://docs.qiime2.org/2018.11/tutorials/moving-pictures/
11. de la Cuesta-Zuluaga J, Escobar J. 2016. Considerations For Optimizing Microbiome Analysis Using a Marker Gene. Front Nutr. 3: 26.
12. Donnenberg M. 2015. 220-Enterobacteriaceae. Mandell, Douglas, and Bennett’s Principles and Practice of Infectious Diseases (Eighth Edition). 2:2503-2517.
13. Cordier J. 2006. 17-Enterobacteriaceae. Emerging Foodborne Pathogens. Woodhead Publishing. p450-475.
14. Suman S, Dhawaria M, Tripathi D, Raturi V, Adhikari D, Kanaujia P. 2016. Investigation of lignin biodegradation by *Trabulsiella* sp. isolated from termite gut. Int Biodeterior Biodegradation. 112: 12-17.
15. Abdullah R, Ananda K, Wijanarka K. 2018. Screening assays of termite gut microbes that potentially as probiotics for human to digest cellulose as new food source. Earth Environ Sci. 157. doi :10.1088/1755-1315/157/1/012027

Appendix A- How to Make Homebrewed Ginger Beer

1. *Ingredients*

: Organic Ginger, Distilled Water, Granulated Sugar, and Lemon Juice

2. *How to Make the "Ginger Bug"*

1) Approximately 40 grams (six tablespoons) of organic ginger were grated.
2) The grated ginger, an equivalent volume of granulated sugar, and 950mL of distilled water were added to a half gallon mason jar and stirred.

Figure S1. Foam Formation on Top of Completed Ginger Bug

3) The center portion of the mason jar lid was replaced with a piece of Parafilm™ that was punctured to allow air through. The lid was screwed on and the mixture sat.
4) After 24 hours, approximately 13 grams of grated and 28 grams of sugar were added to the jar and stirred. The mixture sat for another 24 hours.
5) Step 4 was repeated two more times. The "completion" of the ginger bug can be recognized by the formation of a layer of bubble at the top of the mixture and a rise of the grated ginger to the top (see Figure S1). A light "fizzing" nose may also be heard coming from the bug.

3. *Procedure for Recipe One (used for replicates 1 and 2)*

1) Enough ginger was grated and squeezed to make 64mL of ginger juice.
2) The ginger juice was combined with 3,194mL of distilled water, 27 tablespoons of granulated sugar, 200 mL of lemon juice, and 400 mL of the ginger bug.
3) 355mL of this mixture was poured into each standard size 12oz amber glass bottle (see Figure S2).
4) The bottles were capped and left to ferment.

Figure S2. Replicate one ginger beer bottled and capped

4. Procedure for Recipe Two (used for replicates 3 and 4)

1) Enough ginger was grated and squeezed to make 21mL of ginger juice.
2) The ginger juice was combined with 1000mL of distilled water, 9 tablespoons of granulated sugar, and 50mL of lemon juice to create a "wort".
3) 320mL of the wort was added to each 16oz clear glass swing top bottle.
4) 133mL of ginger bug was added to each bottle.
5) The bottles were sealed and left to ferment.
6) After 6 days, the bottle were moved to a temperature controlled room. The temperature of the room after one day was 74ºF. The day after that, the temperature of the room was 90ºF. Every day after that, the temperature of the room was approximately 82ºF.