Effects of different storage conditions on the development of microorganisms in ground beef

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# Introduction

My primary question was to investigate microbial communities developed in ground beef under different storage conditions. I proposed a hypothesis that more diverse microbial communities would be developed if the meat was stored at room temperature in comparison to the ones kept in a refrigerator as low temperature storage is an essential method to prevent or slow microbial growth (Dave and Ghaly, 2011). I designed my study to compare 3 different ground beef brands in either cooled environment or room temperature. The samples stored in a refrigerator provided distinct colonies for extraction while all the samples stored in room temperature resulted in bacterial lawn. The bacterial lawn prevented DNA extraction process and thus led to skewed results only from the refrigerated samples.

# Methods

## Study design

The model of the experiment was ground beef and they were replicated by purchasing 3 different brands: Teva kosher foods from Trader Joe’s, Lucky California from Lucky Supermarkets, and Butcher shop from Trader Joe’s. The first treatment group remained refrigerated while the second treatment group was stored at room temperature. For both treatment groups, they were stored for 80 hours before subsampled.

## Subsampling

I dipped a sterile cotton swab in PBS buffer first and swabbed each sample on the surface for at least 10 seconds. Then, I broke off the tip of the cotton swab into the labeled tube and closed the cap. To conduct culturing and culture-free experiment for each sample in later steps, I collected total of 18 samples (n = 3 per group, 6 total).

## A. Process of cultured samples

### 1:10/1:100 Dilution and Microbial Culture

I prepared 3 dilutions for each sample: the original stock, 1:10, and 1:100 using PBS buffer (total volume of 200µL each). For culturing process, I transferred 100µL of each diluted sample to 100mm TSA plate and spread the solution with the beads for 10 seconds. The petri dishes were stored in incubator for growth of microbes.

### DNA Extraction and Quantification of DNA concentration

I chose 6 different bacterial morphotypes across the cultured sample and added the cell from colony to a tube using a sterile pipette tip. I performed DNA extraction by following the manufacturer’s protocol from the Sigma REDExtract-N-Amp kit. After the extraction, I measured DNA concentration using Qubit Fluorometer.

### Touchdown PCR

I prepared the master mix for PCR using following ingredients: Amp reagent, 27f primer, 1492r primer, BSA, and water. After aliquoting the master mix and adding DNA to each tube, PCR started with denaturing at 95⁰C for 5 minutes, another denaturing at 94⁰C for 30 seconds, annealing at 65⁰C for 30 seconds, and extending at 72⁰C for 1 minute. The temperature for annealing stepped down 1⁰C per cycle and the second denaturing, annealing,and extending steps were repeated for 10 cycles. Subsequently, additional 25 cycles were started at 94⁰C for 30 seconds, 55⁰C for 30 seconds, and 72⁰C for 1 minute. The cycle stayed at 72⁰C for 10 minutes and went down to 4⁰C for hold.

### Gel Electrophoresis and PCR cleanups

I loaded 6 PCR products and a negative control for 4µL each on 2% agarose gel containing SYBR safe dye and ran the electrophoresis at 140V for 30 minutes. After inspecting the fluorescent bands under UV light, PCR cleanups were performed using Invitrogen Exo-SAP and sent for unidirectional Sanger sequencing using 27f primer at MCLAB in South San Francisco, CA.

### Sanger sequencing and Analysis

I used the software called Geneious Prime to analyze the integrity of the Sanger sequencing data delivered from MCLAB. After reviewing the quality of the samples, I determined 3 usable raw sequences with long stretches of high-quality reads. I trimmed the sequences by removing poor quality reads near the primer site and at the end and manually adjusting bases with IUPAC ambiguity codes. After cleaning the sequences, I aligned the trimmed sequences along with *Thermus aquaticus* as an outgroup. In addition, I used two phylogeny programs, PhyML and MrBayes, within Geneious Prime to construct phylogenetic trees of the aligned sequences. Lastly, I loaded the 3 cleaned sequences and 1 failed sequence into BLAST to identify its gene families.

## B. Process of culture-free samples

### DNA Extraction and Quantification of DNA concentration

I performed DNA extraction by following the manufacturer’s protocol from the Sigma REDExtract-N-Amp kit. After the extraction, I measured DNA concentration using Qubit Fluorometer.

### PCR and Gel Electrophoresis

I prepared the master mix for PCR using following ingredients: Amp reagent, 16SF primer, 16SR primer, BSA, and water. After aliquoting the master mix and adding DNA to each tube, PCR started with denaturing at 95⁰C for 3 minutes. Subsequently, additional 25 cycles were started at 95⁰C for 30 seconds, 55⁰C for 30 seconds, and 72⁰C for 30 seconds. The cycle stayed at 72⁰C for 5 minutes and went down to 4⁰C for hold. 6 PCR products and a negative control were loaded on 2% agarose gel containing SYBR safe dye and ran the electrophoresis at 140V for 30 minutes.

### Illumina sequencing and Analysis

Following electrophoresis, PCR products were purified using AmpureXP magnetic beads and quantitated using a PicoGreen fluorescent assay on a Tecan Infinite M Plex plate reader. Purified PCR products were used as the template for a second round of PCR, which served to attach the Illumina barcodes and adapters. PCR cycles started with denaturing at 95⁰C for 3 minutes followed by additional 25 cycles starting at 95⁰C for 30 seconds, 55⁰C for 30 seconds, and 72⁰C for 30 seconds. The cycle stayed at 72⁰C for 5 minutes and went down to 4⁰C for hold. Following this amplification, PCR products were purified and normalized with a SequelPrep normalization plate, pooled, and then quantified once again with a Qubit 4 fluorometer (Invitrogen) and a TapeStation 4200 (Agilent) to verify library size and concentration. This library was then combined with an Illumina PhiX spike-in library (5% spike in) and sequenced on an Illumina iSeq using a 2 x 150 bp consumable cartridge.

# Results

## A. Cultured samples

### Qubit DNA concentration and Gel Image

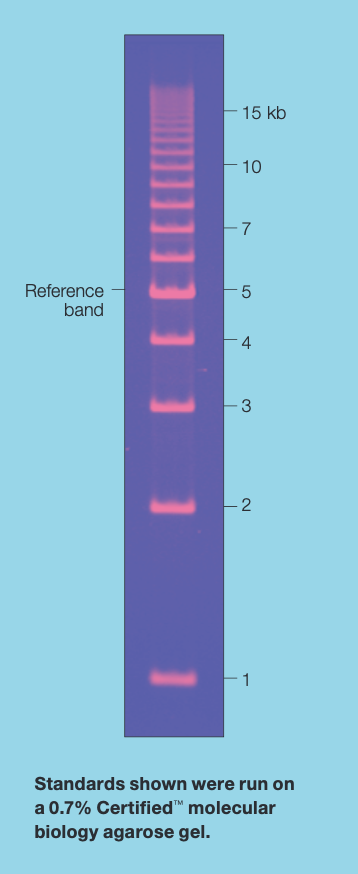
To demonstrate successful DNA extraction, I measured DNA concentration values for each sample using Qubit Fluorometer (Figure 1A). The data validated that all samples contain DNA in it and the values were within 2-30ng/µL range. However, I found no bands in lane 6 and 7, sample #5 and 6 respectively, indicating no DNA separated from the sample (Figure 1B). Sample #1, 2, and 4 showed bright bands while sample #3 produced a faded band. All the visible bands are appeared to be about 1.5kb in fragment size according to EZ Load 1 kb Molecular Ruler (Figure 1C).

|  |  |
| --- | --- |
| Sample ID | DNA Conc. (ng/µL) |
| 1 | 10.50 |
| 2 | 25.50 |
| 3 | 7.56 |
| 4 | 15.10 |
| 5 | 12.40 |
| 6 | 14.30 |

**Figure 1A:** DNA concentration values for culture samples. Sample ID 1 was extracted from the original stock of refrigerated Teva kosher foods from Trader Joe’s, 2 was extracted from 1:100 dilution of refrigerated Teva kosher foods from Trader Joe’s, 3 and 4 were extracted from the original stock of refrigerated Lucky California from Lucky Supermarkets, and 5 and 6 were extracted from the original stock of refrigerated Butcher shop from Trader Joe’s.



**Figure 1B:** Gel image of culture samples. Ladder is shown in lane 1, and the samples from 1 to 6 are located in lane 2-7. Negative control is located in lane 8.



**Figure 1C:** Ladder used in lane 1 (EZ Load 1 kb Molecular Ruler #1708355).

### Comparison of colony abundances and morphotypes

In the boxplot to compare the colony abundances of two treatment groups in 1:10 dilution, the refrigerated samples showed widely spread values while the samples from room temperature treatment group only showed 1,000 abundances with no other variations (Figure 2A). In fact, 1,000 was an arbitrary number to represent the uncountable colony abundance observed from all room temperature samples. No statistical significance was found between the two storage conditions and it failed to reject the null hypothesis (p = 0.20). Similarly in Figure 2B, a broad variation of the colony morphotypes was illustrated in the boxplot for refrigerated samples. The p-value of 0.81 shown in the data indicated no significant difference between the two groups and the p-value close to 1 (0.81) suggested a strong confident that the data is random.



**Figure 2A:** Boxplot of colony abundances at different storage conditions, 10x dilution. Despite a higher median number of colonies from room temperature samples, the sample groups were not significantly different between the two storage conditions (Wilcox p = 0.20).

|  |  |  |  |
| --- | --- | --- | --- |
| statistic | p.value | method | alternative |
| 1.5 | 0.1967056 | Wilcoxon rank sum test with continuity correction | two.sided |



**Figure 2B:** Boxplot showing the number of morphotypes from the two different storage conditions. There was no statistical significance in the morphotypes (Wilcox p=0.81).

|  |  |  |  |
| --- | --- | --- | --- |
| statistic | p.value | method | alternative |
| 5.5 | 0.8136637 | Wilcoxon rank sum test with continuity correction | two.sided |

### Sanger Sequencing and Phylogenetic Trees

After loading the Sanger sequencing data in Geneious Prime, I obtained 3 solid data, 3 completely failed data including the negative control, and 1 seemingly failed data (Figure 3A). I used the 3 successful data after trimming to create Maximum Likelihood and Bayesian phylogenetic trees with *Thermus aquaticus* as an outgroup (Figure 3A, 3B) and found a strong evidence from both phylogenies that support to group Sample #1 and 2 as a monophyletic group (Bayesian posterior probability = 0.9998, ML bootstrap = 98).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| ID | Original File Name | File Name after Correction | Usable? | Length after Trimming | Manually Adjusted Bases |
| 1 | 1-KC\_27f\_A01.ab1 | 1-KC\_27f\_A01\_KC\_cleaned.ab1 | Yes | 611 | 16 |
| 2 | 2-KC\_27f\_B01.ab1 | 2-KC\_27f\_B01\_KC\_cleaned.ab1 | Yes | 713 | 36 |
| 3 | 3-KC\_27f\_C01.ab1 | 3-KC\_27f\_C01\_KC\_failed\_blast.ab1 | No | N/A | N/A |
| 4 | 4-KC\_27f\_D01.ab1 | 4-KC\_27f\_D01\_KC\_cleaned.ab1 | Yes | 552 | 8 |
| 5 | 5-KC\_27f\_E01.ab1 | 5-KC\_27f\_E01\_KC\_failed.ab1 | No | N/A | N/A |
| 6 | 6-KC\_27f\_F01.ab1 | 6-KC\_27f\_F01\_KC\_failed.ab1 | No | N/A | N/A |
| 7 | 7-KC Neg Control | 7-KC Neg Control\_KC\_failed | No | N/A | N/A |

**Figure 3A:** Sanger sequencing data for each sample analyzed by Geneious Prime.



**Figure 3B:** Bayesian phylogenetic tree constructed by MrBayes.



**Figure 3C:** Maximum Likelihood phylogeny constructed by PhyML.

### Identification of Samples using BLAST

I provided 3 successful data (Sample #1, 2, and 4) and 1 seemingly failed data (Sample #3) into BLAST to identify each sample and observed 4 different identifications with strong evidence to support the data (Figure 4).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| ID | Description | % Identity | Query cover | Accession | E-value |
| 1 | *Acinetobacter baumannii* | 97.38 | 100% | CP044356.1 | 0 |
| 2 | *Hafnia paralvei* | 96.36 | 96% | MK995600.1 | 0 |
| 3 | *Kocuria rhizophila* | 90.46 | 77% | KY522908.1 | 5e-136 |
| 4 | *Staphylococcus saprophyticus* | 98.55 | 100% | MH930439.1 | 0 |

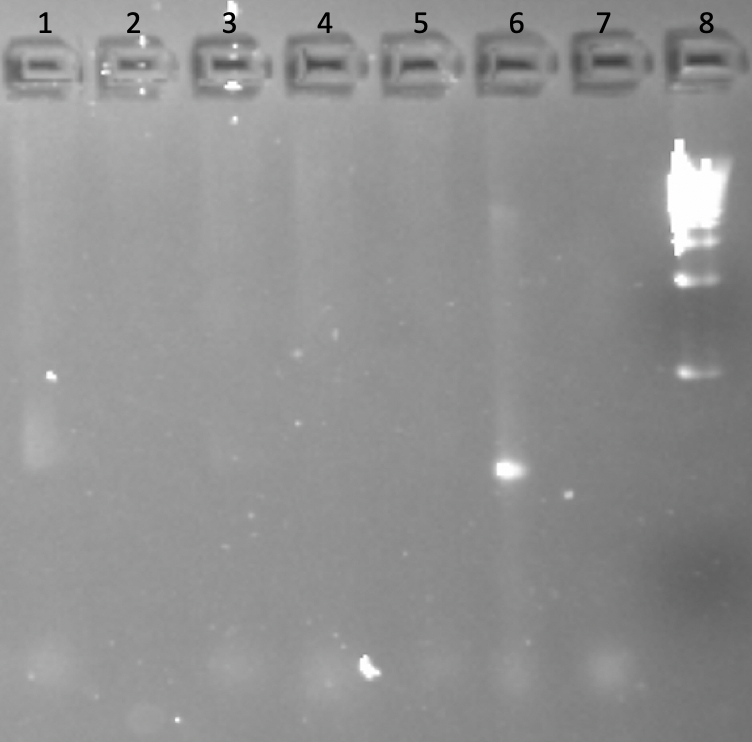
**Figure 4:** Identification of each sample conducted by BLAST.

## B. Culture-free samples

### Qubit DNA concentration and Gel Image

|  |  |
| --- | --- |
| Sample ID | DNA Conc. (ng/µL) |
| 1A | 6.60 |
| 1B | 3.20 |
| 2A | 4.82 |
| 2B | 5.24 |
| 3A | 5.70 |
| 3B | 16.30 |

**Figure 5A:** DNA concentration values for culture-free samples.



**Figure 5B:** Gel image of culture-free samples. Samples from 1A to 3B are located in lane 1-6. Negative control is loaded in lane 7 and the ladder is shown in lane 8.

# Discussion

I questioned a level of microbial communities in ground beef under different storage conditions and expected to observe more diverse communities from the samples stored at room temperature than refrigerated samples. However, the petri dishes of the room temperature samples were covered by bacterial lawn instead of forming colonies and no distinct morphotypes were observed (Figure 4).

The factor that influenced the bacterial lawn in all room temperature samples could be the long swabbing on the surface when subsampled. The highly concentrated stock sample could possibly cause the thick bacterial lawn even though it was diluted down to 1:100. The boxplots in Figure 3 and 4 were displaying data from skewed results due to the unreliable outcomes from room temperature samples. A different approach to enhance the results could be by using streaking method and/or shortening the time when swabbing the sample on the surface.

The gel image from electrophoresis showed no bands for Sample #5 and #6 which corresponded to the failed Sanger sequencing data, and the faded band for Sample #3 corresponded to the seemingly failed Sanger sequencing data. Some possible speculations that influenced no bands for Sample #5 and #6 could be contamination of tubes and/or pipetting errors.

4 different identifications were generated from BLAST for Sample #1, 2, 3, and 4: *Acinetobacter baumannii*, *Hafnia paralvei*, *Kocuria rhizophila*, and *Staphylococcus saprophyticus* respectively (Figure 8). All of the samples except for the complete failures was identified with strong confidence to support the results even for the Sample #3 which showed a faded band on the gel and provided the seemingly failed result from Sanger sequencing data. The results exhibited high percent identity and query coverage values with exceptionally small to zero E-values. However, the phylogenic results that grouped Sample #1 and #2 did not match with BLAST results.

# Sources Cited

Dave,D. and Ghaly,A.E. (2011) Meat spoilage mechanisms and preservation techniques: A critical review. *American Journal of Agricultural and Biological Sciences*, **6**, 486–510.