INTERNATIONAL BACCALAURETE

BIOLOGY EXTENDED ESSAY

THE EFFECTS OF PICKLING METHOD AND TWO WEEKS OF REFRIGERATED STORAGE ON COLONY FORMING UNITS PER MILLILITER OF *LACTOBACILLUS PLANTARUM* IN CABBAGE PICKLES

Research Question: How do colony forming units per milliliter (CFU/mL) of *Lactobacillus plantarum (L. plantarum)* in store-bought, organic fermented and homemade cabbage pickles differ and what effect does refrigerating the pickles at 4°C during 2 weeks of consumption have on the number of these bacteria?

Word Count: 3987

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INTRODUCTION

Cabbage pickles always has a place at our dinner table. My mom says that home-made pickles have good bacteria which support our immunity. Pickling is a method used to increase the shelf life of foods.[1] The oldest method is anaerobic fermentation through immersion of food in brine which allows the microorganisms in food to convert the carbohydrates into acid. The acid produced by lactic acid bacteria (*Lactobacillus*) supresses the food spoilage microorganisms and allows the beneficial bacteria to multiply. The second method is immersion of food in a hot acidic solution involving vinegar which inhibits the spoilage-causing microorganisms.[2] However, the enzymes and the probiotic bacteria are also destroyed. Although this method preserves food, the lack of fermentation means these pickles don't have the same probiotic qualities of fermented pickles.[3]

Cabbage pickle (*Sauerkraut*) is a fermented product with health benefits due to its probiotic content.[4,5] Its fermentation starts with a heterofermentative stage in which *Leuconostoc mesenteroides* begins the fermentation. After 3 to 7 days, homofermentative and acid-tolerant *Lactobacillus* species start to dominate since the pH level decreases below 4.5. *Lactobacillus plantarum* is the most prevalent species at the completion of fermentation.[6]

Probiotic bacteria are defined as "live microorganisms which in adequate amounts confer a health benefit on the host''.[7,8] The number of viable microorganisms is given by colony forming units (CFU). In order to confer benefits, the dose should range between 10^7-10^9 CFU/mL.[9] Microorganisms which are mainly considered as probiotics in cabbage pickles belong to the *Lactobacillus* genus, which includes *L. plantarum* among others.[10-12] These bacteria are also found in the intestinal microbiome and confer health benefits such as prevention and treatment of irritable bowel syndrome,[13-15] cancer prevention,[16,17] reduction of cholesterol,[18,19] and reduction of certain gastrointestinal symptoms.[20,21]

Even in amounts as little as 2 tablespoons, homemade cabbage pickle meets the recommended CFU range in comparison to a supplement probiotic.[22] However, the amount of microorganisms in commercial vinegar-pickled cabbage may be different from the home-made version. Furthermore, since it should be consumed in moderation due to its salt content, the cabbage pickle is generally refrigerated and consumed over several weeks after opening the jar. Since the effects of refrigerated storage and time on the microbial count is unexplored in the literature, I decided to do an experiment to see if pickles made using different methods vary in their number of certain bacteria and if the number of the bacteria are adversely affected during refrigerated storage and gradual consumption. I set my research question as ‘‘**How do colony forming units per milliliter of *Lactobacillus plantarum* in store-bought, organic fermented and homemade cabbage pickles differ and what effect does refrigerating the pickles at 4°C during 2 weeks of consumption have on the number of these bacteria?**’’.

HYPOTHESIS

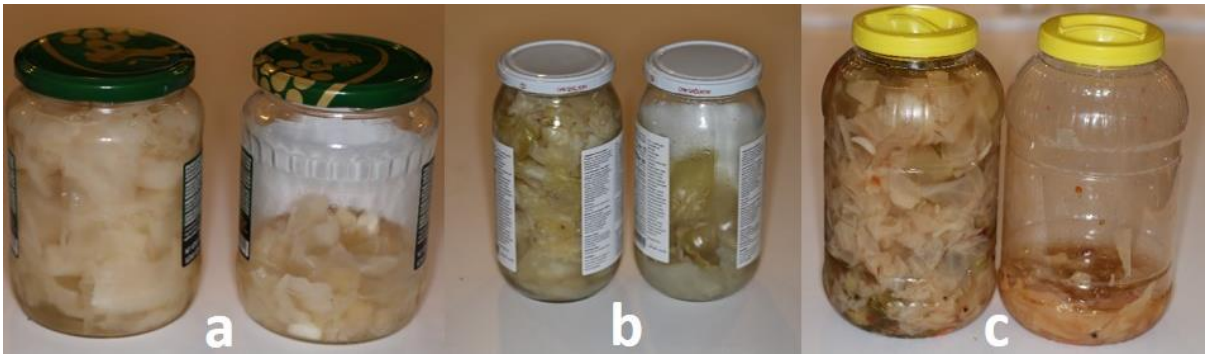
Although there is a decrease in numbers of probiotic bacteria during refrigerated storage of functional food products[23] , milk[24] ,and yoghurt[25] the impact of pickling method and storage on the microbial population of cabbage pickles is not clear. I hypothesized that *L. plantarum* numbers in store-bought cabbage pickles would be lower than those in homemade, and organic fermented cabbage pickles and after two weeks of gradual consumption with refrigerated storage at 4 °C, all types of pickles would exhibit decreased survival of this bacteria.

METHOD DEVELOPMENT AND PLANNING

First, to decide which cabbage pickles to use, I checked our supermarkets or organic food shops. I noticed that cabbage pickles were not always in stock so I decided to obtain the commercial store-bought pickles from Kemal Kükrer and the organic fermented pickles from the Fermented Kitchen online stores. I decided to get the home-made pickles from a family friend who always

prepared her pickles herself and used organic ingredients. The fabrication methods of each type of pickle are given in Appendix I. The store-bought, organic fermented and home-made pickles were in 650 mL, 1000 mL, and 5000 mL jars respectively. (Figure 1a-c) I decided to use a sample size of 10 samples for each pickle type for better accuracy.

Figure 1: Different types of cabbage pickles used in this study at T1 and T2. (a) Store-bought pickles, (b) Organic fermented pickles, (c) Homemade pickles.



I decided to store the pickles in a refrigerator at 4 °C until the beginning of the study. My aim was to slow down further fermentation so that I wouldn't have to open the jars to release the pressure buildup and risk bacterial contamination. When the laboratory became available, I would take the pickles to the laboratory, and obtain my tissue samples. Then I had to close the jars, bring them back home and place them in the refrigerator again. Although the shelf life of refrigerated sauerkraut is long,[26] I knew from personal observation that it takes about 2 weeks (14 days) to finish approximately 1L of pickles for a family of four. In order to mimic this gradual consumption, I decided to take the jars out of the refrigerator, and using a clean fork and spoon for each jar to prevent cross-contamination, take some pickles and pickle juice out of the jar. Since my aim was to simulate the real life, I needed to have one serving of pickle in each jar at the last day of my experiments. Thus I divided the total volume in each jar to 14 days to find the amount of serving per day. This meant that I had to take 46 mL/day, 71 mL/day and 357 mL/day of pickles and brine out of store-bought, organic fermented and home-made pickle

jars respectively. I needed to make sure that the residual pickles were always covered in brine to prevent fungal growth.[27] Then I would put the lid back on and place them back in the refrigerator repeating this process daily for two weeks. I would obtain the measurements at the start of treatment when the jars were first opened (T1) and after 2 weeks of gradual consumption and refrigerated storage (T2) yielding a total of 60 samples.

I also needed to find a suitable method to enumerate the bacteria. There were various methods like the conventional culture method, real-time quantitative polymerase chain reaction (RT qPCR), viability PCR (v-PCR), fluorescent microscopy (FM), MALDI-TOF mass spectrometry, flow cytometry, fluorescence-activated single cell sorting (FACS), and next generation sequencing (NGS).[28,29] Since I couldn't do these on my own due to the necessary equipment, I contacted the researchers at Diagen Biotechnology Research and Development Department. They kindly accepted my using their facilities for this research. (Appendix II). I had access to two methods in this lab which were culture method and qPCR. Although culture method is commonly used in microbiological studies, it is a labor-intensive and time-consuming method which requires a certain skill to interpret the results and may underestimate the number of bacteria since some cells cannot be cultured by culture methods despite being viable.[30] On the other hand, qPCR which is a method that replicates a target segment of DNA millions of times through a thermo-chemical reaction, decreases the risk of cross-contamination and allows for easier, faster, and more accurate quantification of bacteria.[29-32] (Appendix III) (Figure 2) Thus, I decided to use qPCR to quantify *L. plantarum* in my experiments.

When I started researching qPCR, I found out that I needed positive and negative controls for my experiments. The negative control is a sample which contains no DNA. If amplification occurs in this negative control, then this shows contamination occurred. DNase free water is generally used so I decided to use the same. The positive control is a sample with a known amount of DNA which is used to check that the amplification reaction is working. I needed to

do serial dilution in MRS agar to obtain colonies with known amounts of bacteria. However, the lab officials informed me that they didn't allow non-professionals into the area that I needed to use since other sensitive tests were being performed at the same time. Instead, they offered me the use of $7{,}47 \times 10^{-6}$ CFU/mL of *L. plantarum* reference strains from their in-house collection.

Other than the controls, I would also need primers. In qPCR, single-stranded DNA sequences also called oligonucleotides or primers are used to bind with the sample genomic DNA and define the target region that the DNA will be amplified.[33] (Appendix III) These synthetic primers can be designed using special software and fabricated by specialized biotechnology companies. This process required a higher level of education than mine so I decided to use the species-specific PCR primers which had previously been designed for another study in Diagen labs using PrimerBLAST based on recombinase A (recA) gene sequences.[33-35] The 100 mM stock solution of forward and reverse primers in DNase free water were in storage at -20°C at the laboratory. I was informed that *L. plantarum* species specific primer set had a forward primer F: 5'- AGCAGTTCCCTTATCCTT-3' and a reverse primer R: 5'-GAGTGGTTCTTGGTATTCA-3'. I preferred to use a gene-specific primer since it has been shown that this decreases systematic variability of the method.[36]

In order to be able to perform a PCR analysis, I needed to extract DNA from my samples. While researching how to do this, I learned that there are many DNA isolation protocols which can be categorized in 2 groups; chemical-based and solid-phase. Commercially available DNA tissue kits are used together with an automated nucleic acid isolation system and they are based on solid-phase DNA extraction method. Although each method has its own strengths and limitations (Figure 3,Appendix IV), commercially available kits offer a fast, simple and reproducible isolation medium with high quality DNA yield.[37,38] Thus, I decided to use this extraction method.

It has been shown that extracted DNA samples can contain chemicals or excess protein which have the ability to inhibit and reduce sensitivity of the PCR.[39] DNA purity analysis was performed on a spectrophotometer through absorbance measurements with a ratio of absorbance A_{260}/A_{280} used to determine DNA purity since it has been shown that impurities from the DNA extraction protocol reduce this ratio.[40] If the ratio was between 1.7-2.0, the samples were accepted for further analysis.[41] If not, it was taken as indication that contaminants were present and another sample was prepared.

To ensure that my samples were not contaminated and I was not harmed by any of the materials I was working with, I would use sterilized tools, gloves, lab coat and a face shield and work in a disinfected laboratory cabinet. All these materials were kindly provided by Diagen Biotechnology (Appendices I and V). In order to train myself on the procedures, I bought some cabbage pickles and did a preliminary study. I conducted the experiments myself under the supervision of molecular Biologist Mücahit Kaya.

Figure 2: Quantitative PCR Stages and cycle conditions used in this study.[31,32]

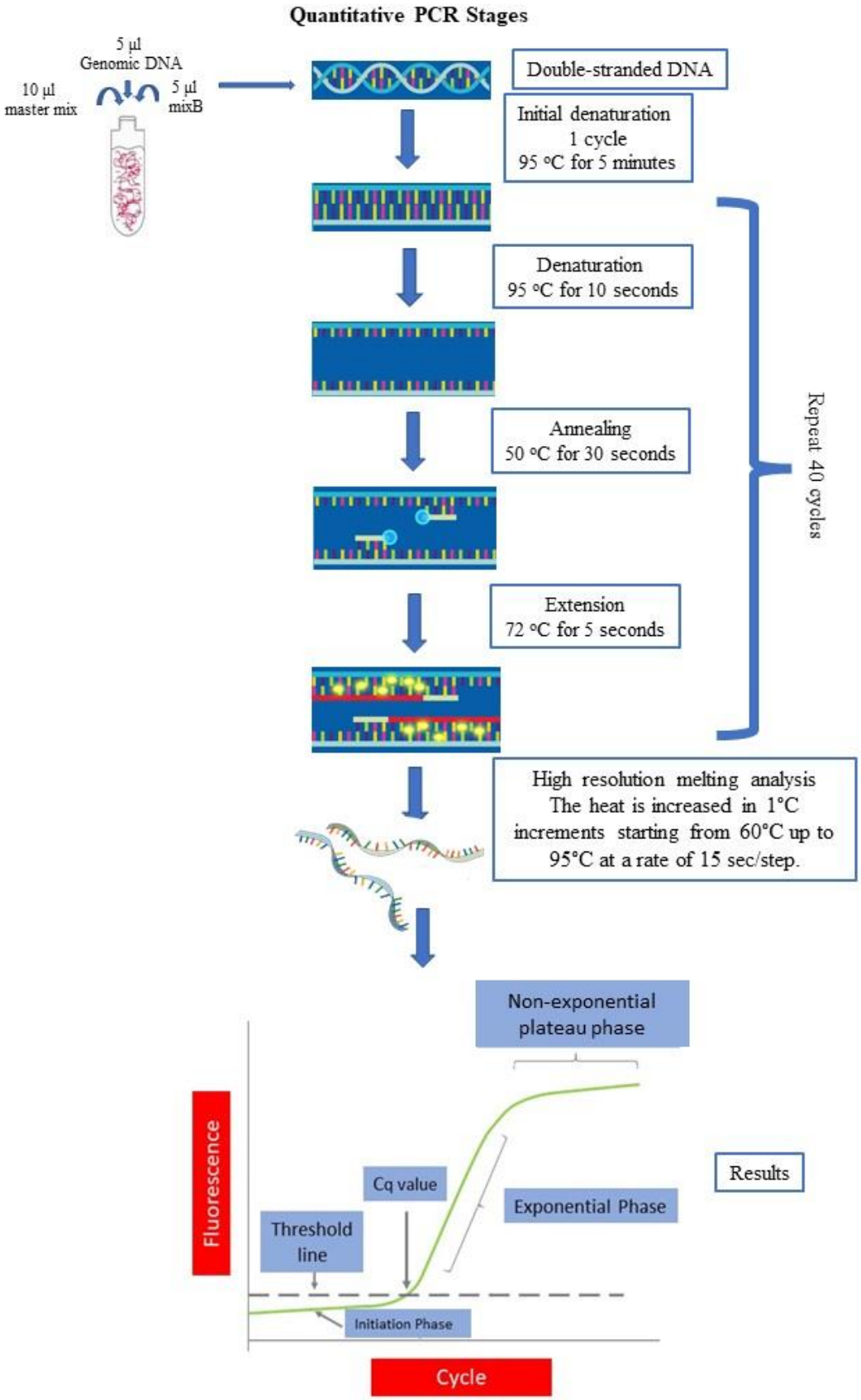
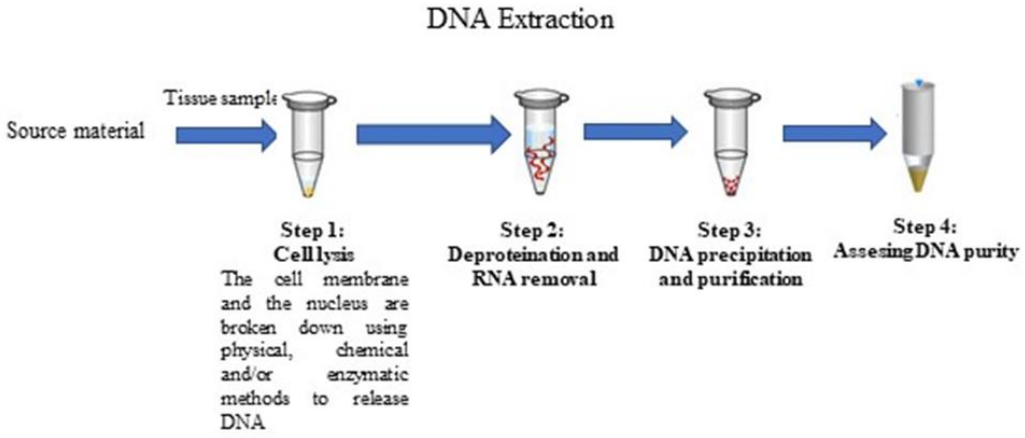


Figure 3: Steps of DNA extraction.[38]



VARIABLES

Independent variable	• Pickles with different preparation methods (store-bought, organic fermented and home-made) • Time (2 weeks)
Dependent variables	• CFU/mL of *L. Plantarum*
Control variables	• DNA extraction method (The same DNA extraction kit was used on all samples) • Mass of pickles and volume of pickle juice (200 µl pickle juice and 25 mg pickle tissue were used in all DNA extraction samples) • qPCR parameters (Same qPCR cycle parameters used in all samples) • Sample storage temperature (Samples stored at 4°C) • Sample volume (20 µl volume of each sample for measurements in qPCR device.) • Same operator (I prepared all the samples) • Room temperature where the samples were prepared (Kept at 22 °C±1 °C with a thermostat)

MATERIALS

Materials	Quantity	Unit(±uncertainty)
Store-bought cabbage pickles	10	650 mL(±1mL)
Organic, fermented cabbage pickles	10	1000 mL(±1mL)
Homemade cabbage pickles	10	5000 mL(±1mL)
Petri dish	30	100X15 mm (±1mm)
Face mask	1	
Medical gloves		
Laboratory apron	1	
DNA tissue kit which included:	1	
• EDT Proteinase K	1	2.5 mL
• MDT Tissue Lysis Buffer	1	25 mL
• LDT Lysis Buffer	1	30 mL
• WDT Wash Buffer	1	160 mL
• CDT Elution Buffer	1	100 mL
• Cartridges	96	
• Collection microtubes	96	1.5 mL
• Caps • Waste tubes	96	
	96	
Screw cap test tubes	75	1.5 Ml
Microcentrifuge tubes	60	0.2 mL
Test tube holders	1	
Dissecting forceps	1	
Zirconia beads	750	1mm(±0.01mm)
Pipette station	1	
Pipettes		

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%99 ethanol	14.4 mL	
Master mix	0.6 mL	
100 mM stock solution of forward and reverse primer for *L. plantarum*	5 µl	
Distilled water	3 L	
DNase free water	250 µl	
Homogenizer	1	
Dry bath incubator	1	
Centrifuge	1	
Vortex Mixer	1	
Automated nucleic acid isolation system	1	
Quantitative PCR cycler	1	

METHOD

1. ***Study Samples and Establishment of Experimental Conditions:***

   1.1 Obtain ten jars each of store-bought, organic fermented and home-made cabbage pickles.

   1.2 Keep them in the refrigerator at 4°C for 2 weeks until the beginning of the experiment.

2. ***Measurements for home-made pickle samples:***

   2.1 Take all the home-made pickle jars out and shake to homogenize the samples.

   2.2 Open all 10 of the home-made pickle jars. The steps described below are repeated for each of the jars to obtain 10 samples.

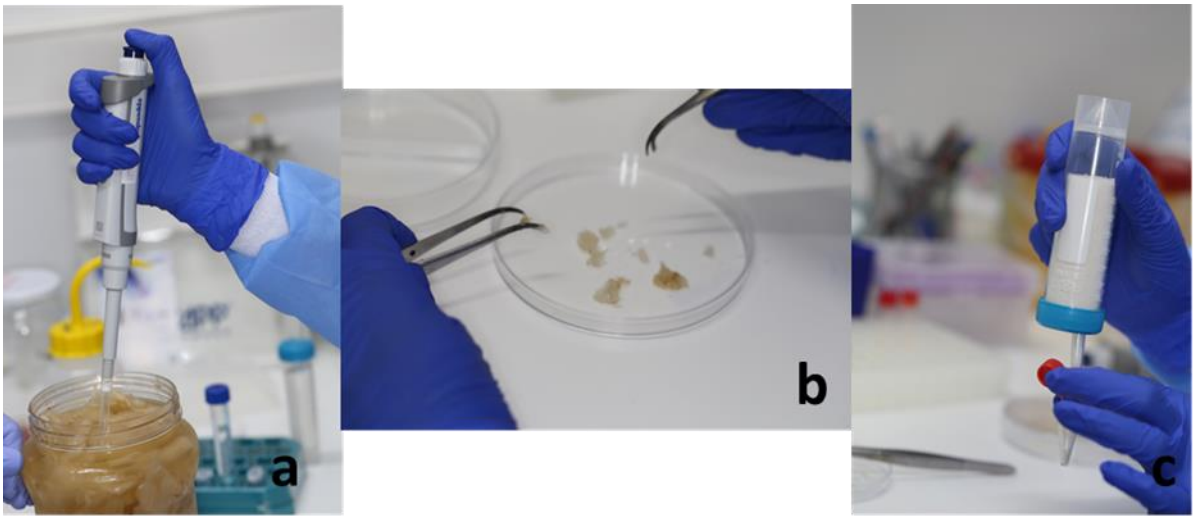
2.3 DNA Extraction:

2.3.1 Using a sterile pipette, place 200 µl pickle juice inside a 1.5 mL homogenization tube.

2.3.2 Using dissecting forceps, take 25 mg pickle tissue, weigh on a a digital scale, and divide it into pieces.

2.3.3 Add 250 µl MDT (Tissue Lysis Buffer) and ten zirconia beads with a diameter of 1.0 mm and homogenize by mixing the sample 2 times for 20 seconds at 5000 rpm to break down the tissue. (Figure 4a-c).

Figure 4: Steps of sample preparation. (a) Extraction of 200 µl pickle juice by pipette, (b) Dissection of pickle tissue, (c) Addition of zirconia beads.

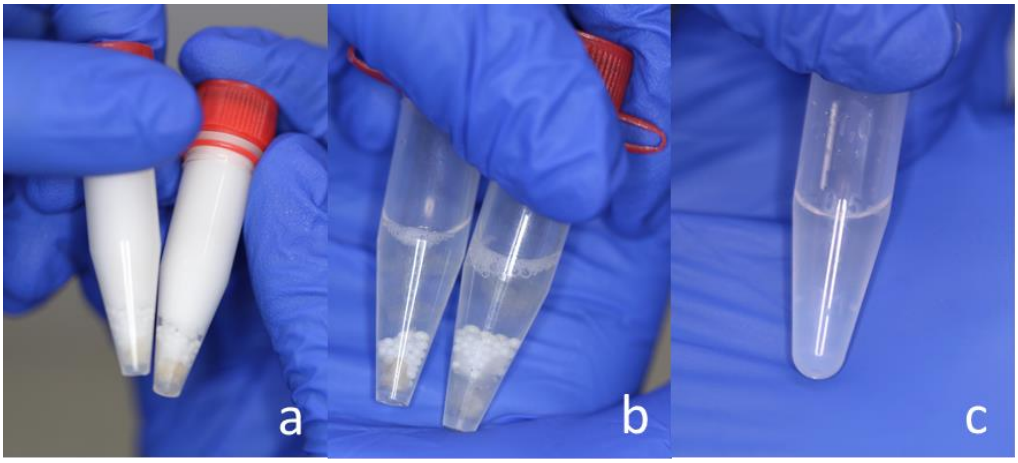


2.3.4 After homogenization (Figure 5a), add 25 µl EDT (Proteinase K) solution and incubate at 56°C for 60 minutes, centrifuge for 10 minutes at 15000g at room temperature to obtain supernatant.

2.3.5 Transfer supernatant (Figure 5b) to 1.5 mL of microtube, add 180 µl LDT (Lysis Buffer), vortex for 15 seconds, and incubate for 10 minutes at 70°C. Add 240 µl %99 cold ethanol and vortex for 15 seconds to obtain lysate.

2.3.6 Transfer lysate (Figure 5c) to automated nucleic acid isolation system cartridges and apply air pressure. Wash the remaining material in the tube for 3 cycles by adding 750 µl WDT (wash buffer) and applying pressure. After the third cycle, place the cartridge holder into the elution position of the machine in order to release the purified DNA into the elution buffer. Use 200 µl of CDT (elution buffer) for elution. Incubate for 90 seconds at room temperature and apply air pressure again. Add 100 µl CDT to achieve a solution with 50-60 ng genomic DNA.

Figure 5: Steps of lysate preparation for DNA extraction. (a) Homogenized sample, (b) Supernatant, (c) Lysate.



2.4 DNA Purity Analysis:

2.4.1 Perform DNA purity analysis using a spectrophotometer through absorbance measurements by putting a drop of sample onto the measuring pedestal, closing the lid and clicking measure. If the ratio of absorbance A_{260}/A_{280} is between 1.7-2.0, accept the samples for analysis. If not, prepare another sample.

2.5 qPCR Analysis Procedure:

2.5.1 Thaw the 100 mM stock solution of forward and reverse primers in DNase free water, which were in storage at -20°C at the laboratory, at 4°C.

2.5.2 Prepare 250 µl of mixB solution by adding 5 µl of forward primer, 5 µl of reverse primer and 240 µl of DNase free water to 1.5 mL microtube.

2.5.3 For each sample, add 10 µl master mix, 5 µl mixB which includes 0.5mM forward and 0.5mM reverse primer and 5 µl gDNA to prepare a 20 µl sample in 0.2 mL microcentrifuge tubes.

2.5.4 Place the sample tubes, negative control and positive control in the qPCR device. Set the test conditions to replicate the fragments at first denaturation for 1 cycle at 95 ^{o}C for 5 minutes, 40 cycles of denaturation at 95 ^{o}C for 10 seconds, annealing at 50 ^{o}C for 30 seconds, and extension at 72 ^{o}C for 5 seconds.

2.5.5 After the amplification steps are concluded, generate high resolution melt curves of the samples by increasing the heat in 1°C increments starting from 60°C up to 95°C at a rate of 15 sec/step.

2.5.6 After qPCR is finished, the software calculates the number of colony forming units (CFU) per mL of prepared sample by correlating the cycle threshold values with positive controls. Record these measurements as T1 values for home-made pickles.

3. Measurements for store-bought pickle samples:

3.1 Take all the store-bought pickle jars out and shake to homogenize the samples.

3.2 Open all 10 of the store-bought pickle jars. Repeat steps 2.3, 2.4 and 2.5 for each of the store-bought pickle jars.

4. Measurements for organic fermented pickle samples:

4.1 Take all the organic fermented pickle jars out and shake to homogenize the samples.

4.2 Open all 10 of the organic fermented pickle jars. Repeat steps 2.3, 2.4 and 2.5 for each of the organic-fermented pickle jars.

5. Keep the jars in the refrigerator at 4°C while taking 46 mL, 71 mL and 357 mL of pickles and brine out of store-bought, organic fermented and home-made pickle jars respectively every day for 2 weeks.
6. After 2 weeks, repeat steps 2, 3 and 4 to obtain T2 measurements.

DATA COLLECTION AND PROCESSING

Table 1. Raw data of CFU/mL of *L. plantarum* in the store-bought, organic fermented and home-made cabbage pickles at T1 (first time the jar is opened) and T2 (2 weeks of refrigerated storage at 4°C during gradual consumption). The numbers are given in scientific notation format yE+x which means "y multiplicated by 10 to the power of +x ($y*10^{+x}$)".

Groups	**Trials**	*L. plantarum* (CFU/mL)			Storage temperature (°C ±1°C)	Sample volume (µL ±0.2µL)	Room temperature (°C ±1°C)
		Store bought pickles	Organic fermented pickles	Home-made pickles			
T1	1	1.15E+03	9.56E+05	6.69E+04	4	20.0	22
	2	2.46E+03	4.28E+05	2.15E+05	4	20.0	22
	3	2.95E+02	8.45E+05	1.08E+05	4	20.0	22
	4	9.83E+03	1.08E+06	9.49E+04	4	20.0	22
	5	2.26E+03	7.61E+05	8.26E+04	4	20.0	22
	6	7.43E+03	8.64E+05	2.26E+05	4	20.0	22
	7	5.36E+03	9.94E+05	3.43E+05	4	20.0	22
	8	1.29E+04	1.59E+06	1.78E+05	4	20.0	22
	9	8.56E+03	8.73E+05	2.47E+05	4	20.0	22
	10	1.65E+04	9.65E+05	9.23E+04	4	20.0	22
T2	1	1.97E+03	7.38E+05	5.54E+04	4	20.0	22
	2	1.08E+03	4.02E+05	2.03E+05	4	20.0	22
	3	9.62E+01	7.78E+05	8.72E+04	4	20.0	22
	4	2.41E+03	8.29E+05	9.02E+04	4	20.0	22
	5	8.49E+02	6.67E+05	6.19E+04	4	20.0	22
	6	8.92E+03	7.51E+05	1.58E+05	4	20.0	22
	7	2.95E+03	9.21E+05	1.44E+05	4	20.0	22
	8	9.23E+03	1.27E+06	1.02E+05	4	20.0	22
	9	7.29E+03	5.92E+05	1.35E+05	4	20.0.	22
	10	1.08E+04	9.12E+05	1.01E+05	4	20.0	22

- Mean was calculated:

$$\text{Mean } (\bar{x}) = \frac{\sum x}{n}$$

x: measured value

Σx: sum of observed values

n: number of observations.

Example calculations made with store-bought pickle group at T1:

$$\sum x = (1.15E + 03) + (2.46\text{E} + 03) + (2.95\text{E} + 02) + (9.83\text{E} + 03) + (2.26\text{E} + 03) + (7.43\text{E} + 03)$$
$$+ (5.36\text{E} + 03) + (1.29\text{E} + 04) + (8.56\text{E} + 03) + (1.65\text{E} + 04) = 6.67\text{E} + 04$$

$$\frac{\sum x}{10} = \frac{6.67E + 04}{10} = 6.67E + 03$$

- Standard deviation was calculated:

$$s = \sqrt{\frac{\sum_{i=1}^{N}(x_i - \bar{x})^2}{N - 1}}$$

s: the sample Standard Deviation

N: number of observations

X_i: value of each observation

$\bar{x}$: the sample mean

Standard deviation for the example above:

Observed value	Mean	Difference	Squared difference
1.15E+03	6.67E+03	-5.52E+03	3.05E+07
2.46E+03	6.67E+03	-4.21E+03	1.77E+07
2.95E+02	6.67E+03	-6.38E+03	4.06E+07
9.83E+03	6.67E+03	3.16E+03	9.99E+06
2.26E+03	6.67E+03	-4.41E+03	1.94E+07
7.43E+03	6.67E+03	7.60E+02	5.78E+05
5.36E+03	6.67E+03	-1.31E+03	1.72E+06
1.29E+04	6.67E+03	6.23E+03	3.88E+07
8.56E+03	6.67E+03	1.89E+03	3.57E+06
1.65E+04	6.67E+03	9.83E+03	9.66E+07

$$\sum_{i=1}^{N}(x_i - \bar{x})^2 = (3.05E+07) + (1.77E+07) + (4.06E+07) + (9.99E+06) + (1.94E+07)$$

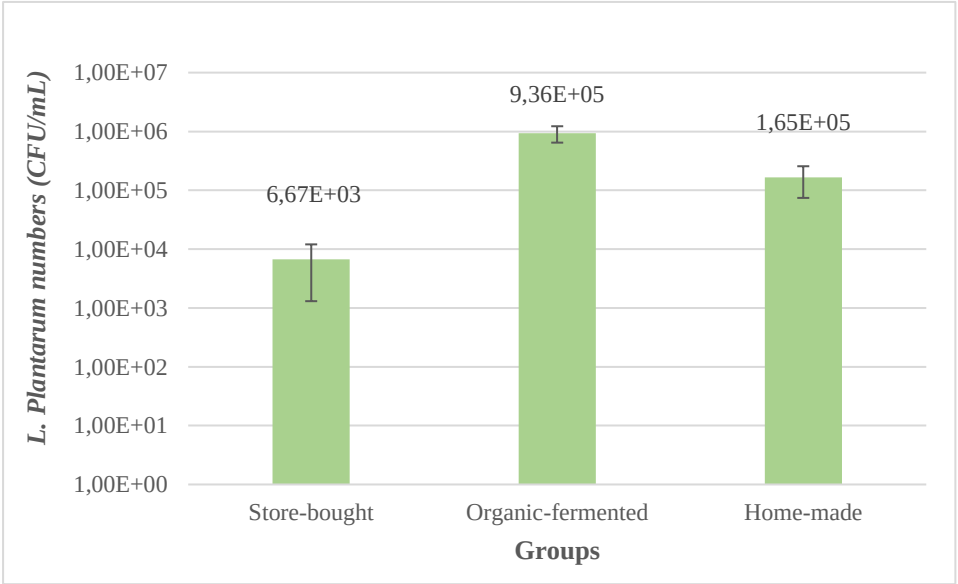
$$+ (5.78E+05) + (1.72E+06) + (3.88E+07) + (3.57E+06) + (9.66E+07)$$

$$= 2.60E+08$$

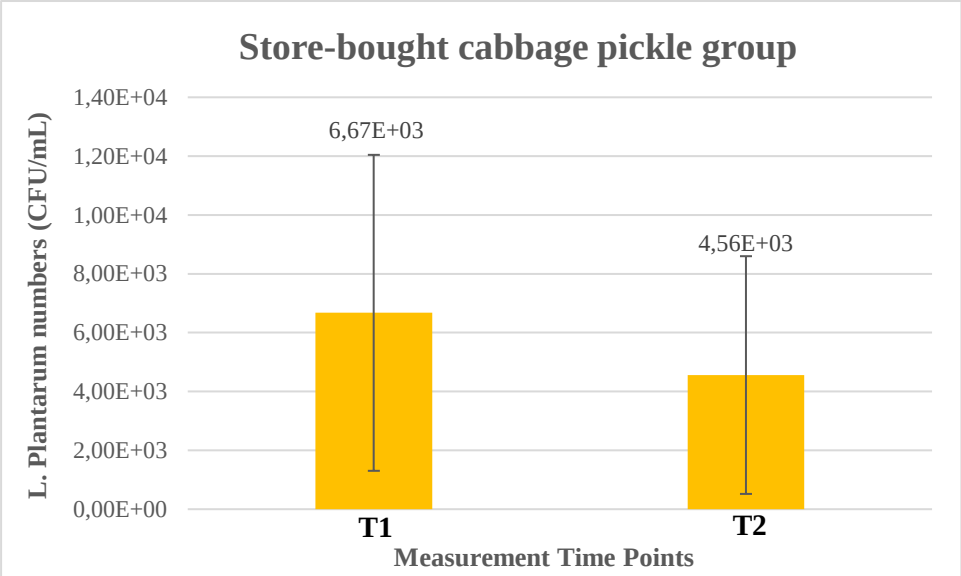
$$\sqrt{\frac{\sum_{i=1}^{N}(x_i - \bar{x})^2}{N-1}} = \sqrt{\frac{2.60E+08}{9}} = 5.37E+03$$

Table 2. Descriptive statistics of CFU/mL of *L. plantarum* in the store-bought, organic fermented and home-made cabbage pickles at T1 and T2 (2 weeks of refrigerated storage at 4°C during gradual consumption). The numbers are given in scientific notation format yE+x which means "y multiplicated by 10 to the power of +x ($y*10^{+x}$)".

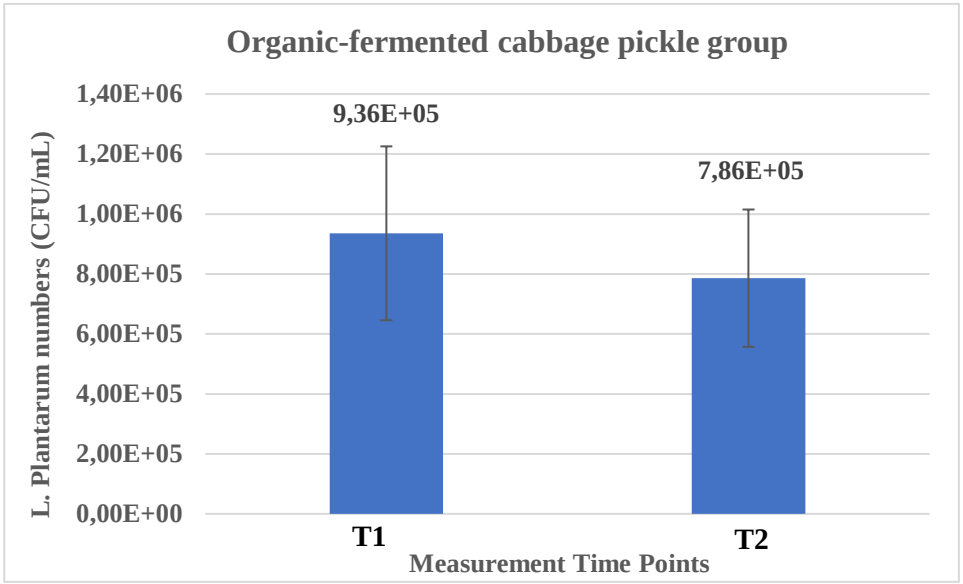
Variable	Group	Time	Number of samples	Mean	Median	Standard Deviation	Standard Error	Variance	95% confidence interval
L. plantarum (CFU/mL)	Store bought	T1	10	6.67E+03	6.40E+03	5.37E+03	1.70E+03	2.88E+07	3.84E+03
		T2	10	4.56E+03	2.68E+03	4.04E+03	1.28E+03	1.63E+07	2.89E+03
	Organic-fermented	T1	10	9.36E+05	9.15E+05	2.90E+05	9.18E+04	8.44E+10	2.08E+05
		T2	10	7.86E+05	7.65E+05	2.29E+05	7.25E+04	5.26E+10	1.64E+05
	Home-made	T1	10	1.65E+05	1.43E+05	9.11E+04	2.88E+04	8.30E+09	6.52E+04
		T2	10	1.14E+05	1.02E+05	4.59E+04	1.45E+04	2.10E+09	3.28E+04



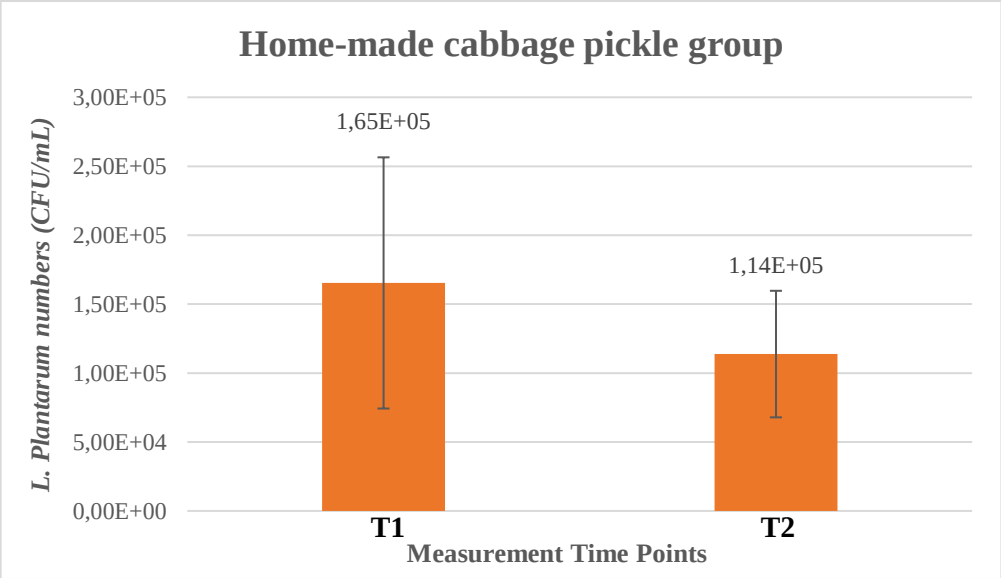
Graphic 1. Processed bar-graph of means of *L. plantarum* numbers in the store-bought, organic fermented and home-made cabbage pickles at T1. Error bars were added using standart deviation values.



Graphic 2. Processed bar-graph of means of *L. plantarum* numbers in the store-bought cabbage pickles at T1 and at T2. Error bars were added using standart deviation values.



Graphic 3. Processed bar-graph of means of *L. plantarum* numbers in the organic fermented cabbage pickles at T1 and at T2. Error bars were added using standart deviation values.



Graphic 4. Processed bar-graph of means of *L. plantarum* numbers in the home-made cabbage pickles at T1 and at T2. Error bars were added using standart deviation values.

STATISTICAL ANALYSIS

My hypotheses and my null hypotheses which were being tested by statistical analyses were as follows:

Hypothesis 1:

H_1:There is a statistically significant difference in *L. plantarum* numbers between store-bought, homemade and organic-fermented cabbage pickle groups.

H_0: There is no statistically significant difference in numbers of *L. plantarum* colonies of store-bought, homemade, and organic-fermented cabbage pickle groups.

Hypothesis 2:

H_1: There is a significant decrease in the numbers of *L. plantarum* after two weeks of gradual consumption with refrigerated storage at 4 °C in all groups.

H_0: There is no statistically significant difference in numbers of *L. plantarum* at the beginning and after two weeks of gradual consumption with refrigerated storage at 4 °C.

In order to test my hypotheses, I used One-way ANOVA analysis. (Table 3)

Table 3. The results of One-way ANOVA analysis. The numbers are given in scientific notation format yE+x which means "y multiplicated by 10 to the power of +x ($y*10^{+x}$)" or yE-x which means "y multiplicated by 10 to the power of -x ($y*10^{-x}$)".

Dependent Variable	**Result Details**					
L. plantarum	***Source***	***SS***	***df***	***MS***	***F***	***p-value***
	Between-treatments	8,58826E+12	5	1,71765E+12	F=69,9370621	2,36788E-22*
	Within-treatments	1,32624E+12	54	24559976618		
	Total	9,9145E+12	59			

*Statistically significant difference with $p<0.05$, H_0 for Hypotheses 1 and 2 were rejected.

Since the ANOVA analysis showed significant differences between groups, further analysis using independent samples t-test assuming unequal variances was done to compare the measurements at T1 to see which groups were significantly different from each other (Table 4).

Table 4. Independent sample t-test analysis of *L. plantarum* at T1 between groups. The numbers are given in scientific notation format yE+x which means "y multiplicated by 10 to the power of +x ($y*10^{+x}$)" or yE-x which means "y multiplicated by 10 to the power of -x ($y*10^{-x}$)".

	L. plantarum at T1 (store-bought)	*L. plantarum* at T1 (organic-fermented)		*L. plantarum* at T1 (store-bought)	*L. plantarum* at T1 (home-made)		*L. plantarum* at T1 (organic-fermented)	*L. plantarum* at T1 (home-made)
Mean	6,67E+03	9,36E+05		6,67E+03	1,65E+05		9,36E+05	1,65E+05
Variance	2,88E+07	8,44E+10		2,88E+07	8,30E+09		8,44E+10	8,30E+09
Observations	10	10		10	10		10	10
Hypothesized mean difference	0			0			0	
df	9			9			11	
t Stat	-10,11244062			-5,500385583			8,002048542	
P(T<=t) one-tail	1,63E-06			0,000189998			3,25817E-06	
t Critical one-tail	1,833112933			1,833112933			1,795884819	
P(T<=t) two-tail	3,25999E-06*			0,000379996*			6,51633E-06*	
t Critical two-tail	2,262157163			2,262157163			2,20098516	

*Statistically significant difference with $p<0.05$in all t-tests. H_1 of Hypothesis 1 was supported since all groups showed significant differences at T1.

To test Hypothesis 2, I compared the measurements at different time points in the same group using dependent-samples t-test (Table 5).

Table 5. Dependent sample t-test analysis of *L. plantarum* numbers between time points. The numbers are given in scientific notation format yE+x which means "y multiplicated by 10 to the power of +x ($y*10^{+x}$)".

	L. plantarum numbers at T1 (store-bought)	*L. plantarum* numbers at T2 (store-bought)		*L. plantarum* numbers at T1 (organic-fermented)	*L. plantarum* numbers at T2 (organic-fermented)		*L. plantarum* numbers at T1 (home-made)	*L. plantarum* numbers at T2 (home-made)
Mean	6.67E+03	4.56E+03		9.36E+05	7.86E+05		1.65E+05	1.14E+05
Variance	2.88E+07	1.63E+07		8.44E+10	5.26E+10		8.30E+09	2.10E+09
Observations	10	10		10	10		10	10
Pearson Correlation	0.860173917			0.942169683			0.750487533	
Hypothesized mean difference	0			0			0	
df	9			9			9	
t Stat	2.389096467			4.422320957			2.539597524	
P(T<=t) one-tail	0.020308753			0.000832788			0.015865865	
t Critical one-tail	1.833112933			1.833112933			1.833112933	
P(T<=t) two-tail	0.040617506*			0.001665576*			0.031731729*	
t Critical two-tail	2.262157163			2.262157163			2.262157163	

*Statistically significant difference with $p<0.05$ in all t-tests. H_1 of Hypothesis 2 was supported and H_0 of Hypothesis 2 was rejected since there is statistically significant difference between T1 and T2 in all pickle groups.

ANALYSIS AND DISCUSSION

Since *L. plantarum* numbers were significantly different between groups (all p values less than $\alpha = 0.05$) (Tables 3 and 4, Graphic 1), H_1 of Hypothesis 1 was supported and H_0 of Hypothesis 1 was rejected. The finding that different cabbage pickles differ in their microbial community and that store-bought products contain little amount of bacteria due to pasteurization is in agreement with other studies.[22,42,43] The microbial variety of a fermented product is dependent on the materials used, the process of fermentation and the environmental conditions.[42] Since the probiotic bacterial count should be between 10^7-10^9 CFU/mL to have health benefits, it is important to know the microbial community counts of a product we are consuming.[9] In my

study, I only enumerated one of the probiotic bacteria (*L. plantarum*) in different types of cabbage pickles which is why I cannot deduce whether the total microbial count will be between 10^7-10^9 CFU/mL in my samples. However, considering that there are many other bacteria in cabbage pickles,[6] it can be assumed that the total microbial community counts could be in that range, especially in the organic fermented (9.36E+05 CFU/mL) and home-made (1.65E+05 CFU/mL) cabbage pickle groups with higher numbers of *L. plantarum* (Table 2, Graphic 1). On the other hand, the numbers of probiotic bacteria *L. plantarum*, which is the dominant bacteria in cabbage pickles,[6] in store-bought (6.67E+03 CFU/mL) group was significantly lower than the other groups with numbers at the 10^3 CFU/mL range (Tables 2,3 and 4; Graphic 1), which could mean that the total bacterial counts may not reach the values which confer health benefits.

The decrease in *L. plantarum* numbers from T1 to T2 in store-bought (from 6.67E+03 to 4.56E+03 CFU/mL, Graphic 2), organic fermented (from 9.36E+05 to 7.86E+05 CFU/mL, Graphic 3) and home-made (from 1.65E+05 to 1.14E+05 CFU/mL, Graphic 4) pickles were significant in all groups (Table 5). Thus H_1 of Hypothesis 2 was supported and H_0 of Hypothesis 2 was rejected. A sauerkraut should have a pH of 3.5 or less after fermentation due to 1.8–2.3% lactic acid content.[44] *L. plantarum* is primarily responsible from this acidic environment which prevents the growth of other microorganisms which cause food spoilage or illness in humans.[45,46] In this study, as their numbers decreased over time, this could have caused the acidity of the environment to decrease as well. In that case, acid intolerant bacteria such as *Salmonella or* coliform bacteria such as *E. Coli* could reproduce causing a danger to those who consume these products.[46,47]

Similar to our results, other researchers also report that *L. plantarum* have decreased survival rates during storage, even when refrigerated.[48-51] However, this may not be indicative of a

decrease in all probiotic bacteria over time since the adaptation of various species of lactic acid bacteria to their ecosystems are different which reflects their different stress responses.[52]

EVALUATION

The standard deviations relative to the mean values for *L. plantarum* numbers for organic fermented (T1:9.36E+05±2.90E+05, T2:7.86E+05±2.29E+05) and home made pickle (T1: 1.65E+05±9.11E+04, T2: 1.14E+05±4.59E+04) groups were low, whereas they were higher for the store-bought (T1: 6.67E+03±5.37E+03, T2: 4.56E+03± 4.04E+03) group. This means the *L. plantarum* numbers are farther away from the mean in the store-bought group whereas they are relatively closer to the mean in the organic fermented and home-made groups.

There are some limitations of this study. The sizes of jars and the mass of pickles inside them were different from each other which may have affected the number of the bacteria. Standardizing the volume of jars and pickle mass in further studies can control for this variable and increase the reliability of the results.

I chose the average consumption time of 2 weeks using my own family as an example. However others may consume the pickles in shorter or longer durations. Planning further studies to reflect these differences will help us better understand the effects of time on the viability of probiotic bacteria.

I used manual pipetting to prepare the samples. It has been shown that there is considerable imprecision during dispension of fluids with this method.[53] This may have introduced systematic and random error to sample preparation. Using automatic pipettes may help decrease error during sample preparation.

Another limitation of the study is that the salt content and the pH of the groups are not known. It has been shown that the lactic acid bacteria are affected by the differences in salinity and acidity with 2-4% salt and a pH of 3.5 or less being ideal.[44,54] If the salinity or the pH of the

groups are different, this may have created different environmental conditions for the viability of the bacteria. Choosing pickles with the same amount of salt and pH for the study may yield more comparable results.

Despite these limitations, this was a highly standardized experiment. Since most of the variables were controlled for, it is possible to replicate the experiment under the same conditions and validate the reliability of the results. Another strength of the study was the use of qPCR to enumerate bacteria since this method is highly sensitive and specific and eliminates intra- and inter-examiner reliability problems by automatic counting.[55] The number of samples in each group was also high, which yielded 60 observations at the end of the study. This high number of 10 trials per group increases the power of the study and makes the study less prone to error.

CONCLUSION

Consuming cabbage pickles appears to be good for incorporating probiotic bacteria to a daily diet. The highest numbers of *L. plantarum* colonies were found in organic fermented and home-made cabbage pickles while store-bought pickles had very low numbers. Viability of *L. plantarum* in cabbage pickles decreases over time. According to the results of this study, organic fermented cabbage pickles offer the best natural alternative for ingestion of probiotic bacteria. Although much research has been done to determine the probiotic potential of foods including cabbage pickles, the effects of storage and consumption conditions are not well documented. Isolating different bacteria and quantifying their long-term survival in different types of pickles will let us better understand the beneficial health effects of pickles in general. A new research question which can be addressed in further investigations is: ‘‘ How do numbers of *L. acidophilus* and *L. brevis* colonies in cucumber and pepper pickles differ and what effect does unrefrigerated storage for a long term of 3 months have on the number of these bacteria?’’

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APPENDICES

Appendix I: Pickling methods used in the study

Store-bought cabbage pickles: Fresh cabbage is harvested during season, washed and transferred to fermentation tanks with brine. It is allowed to ferment at ambient temperature for 1 month in a closed tank. During fermentation period, the mixture is circulated and tanks are appropriately maintained. After fermentation is complete, the cabbages are washed, selected, rewashed, chopped and rewashed before they are filled into jars. 65-75°C brine is poured to cover the cabbages in the jar and the lids are sealed. When the jars cool down to 55°C, they are submerged in hot water with a temperature of 85-90 °C for 25-30 minutes for pasteurization and then cooled again. This hermetically seals the lids and the product is kept fresh.

Organic fermented cabbage pickles: The organic cabbages harvested during season are washed, chopped, and pressed into jars. Using 3 table spoons of grounded rock salt for every 1L of drinking water a brine is prepared. This brine is poured into the jar and weighted down until the cabbages are fully submerged. The jars are stored in a dark place at room temperature. After 7-10 days the pickle is ready for consumption.

Home-made cabbage pickles: 5L jars are washed thoroughly, and dried. Cabbages from the farmer's market are washed and chopped in a large container. 3-4 table spoons of grounded rock salt is sprinkled on the cabbages and they are mixed until the cabbages release their water. Bay leaves and black pepper seeds are put in a 5L jar and cabbages are put on top of these until the jar is half full. This process is repeated until the jar is filled. 2L drinking water, 1 cup grounded rock salt, 1 cup homemade vinegar, juice of 1 lemon and a pea-sized lemon salt are mixed well to create brine. This brine is poured into the jar and 2 wooden skewers are placed diagonally to press the cabbages under the brine. The lid is sealed and the jars are kept in a dark and cold place. The pickle is ready to eat in 2 weeks.

Appendix II: Diagen Biotechnology confirmation letter

March 22,2021

To Whom It May Concern,

This letter is written to confirm the support of Diagen Biotechnology for the research project by [redacted]

[redacted] requested our permission to use our laboratory facilities for her research which will be conducted using plant-based tissue. We hereby confirm that she has been granted permission to use our laboratory. She will have authorization to use the laboratory equipment and the materials needed for her study. She will do all her experiments by herself under the supervision of our Molecular Biologist, Mücahit Kaya . She is also allowed to use the reference strains and species-specific PCR primers from our culture collection needed for her experiments.

Please do not hesitate me if you need any further information.

Yours sincerely,

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Appendix III: Information about qPCR*

Polymerase chain reaction (PCR) is a rapid, simple, and culture-independent technique used to replicate target segments of DNA through DNA denaturation, primer annealing, and extension stages using thermo-chemical reactions.[31]

Initial Denaturation Stage: During the denaturation stage, the double-stranded DNA is seperated into two single strands by heating the sample between 94-98°C to break the hydrogen bonds. After an initial denaturation of long duration for one time, thermal cycling of denaturation, annealing and extension is established.

Denaturation Stage: During this first step of the repeat cycle the sample is heated between 94-98°C for 10-60 seconds to further seperate the two strands of DNA.

Annealing Stage: Annealing is the binding of primers to target sequences on the single DNA strands. These primers are species-specific oligonucleotides which are used to bind to the sample denatured DNA and define the target segment of the DNA to be amplified. The forward primer binds to the 3' to 5' running bottom strand while the reverse primer binds to the strand above that runs 5' to 3'.[35] For annealing, the sample is cooled to between 45-65°C for 15-60 seconds.

Extension Stage: At the final stage of extension, the sample is once again heated between 70-80°C for 5-30 seconds to allow the binding of nucleotides with the previously annealed primer which makes copies of the target DNA, which is called an amplicon.

Up to 40 repeated cycles of these processes in a thermocycler machine leads to over a billion copies of the original DNA segment in just a few hours. After these cycles are finished, high resolution melt curves can be generated to check for artifacts and reaction specificity by heating the sample in small increments back to denaturation temperature values.

The invention of PCR was considered to be one of the most important scientific advances in molecular biology and its inventor won the Nobel Prize for Chemistry.[32] The concept of using fluorescent dyes to quantify amplicons in real time, which is also called quantitative PCR (qPCR), was another important breakthrough. In qPCR, the intensity of fluorescence is determined in each PCR reaction cycle and the point this intensity reaches above background level is called quantification cycle (C_q). The C_q value is used to determine the amount of target DNA in a sample in relation to standard samples. If the C_q value is lower, it means that amplification is observed in earlier stages and there is more target DNA.

*Also see Figure 2 for the qPCR cycle conditions used in this study.

Appendix IV: Information about DNA extraction*

DNA extraction is a process used to isolate high-quality DNA from biological samples. Although there are different DNA extraction methods, the steps of DNA extraction are similar between methods:[37,38]

1. Cell Lysis

The first step is to disrupt the cellular structure to release nucleic acid into a solution called lysate. There are different methods for lysis: physical, enzymatic, chemical and a combination of the three. During physical methods, the tough cell wall is crushed using freze-grinding, beads or sonic devices. In chemical or enzymatic methods, various detergents, chemical solutions or enzymes such as Proteinase K are used to disrupt the cellular structure. Most DNA extraction methods use a combination of these three during their protocols.

2. Deproteination and RNA removal

After the lysate is prepared, it should be cleared of any cellular structures, proteins or chemicals before the DNA can be purified further. This is usually achieved by centrifugation or filtration.

3. DNA precipitation and purification

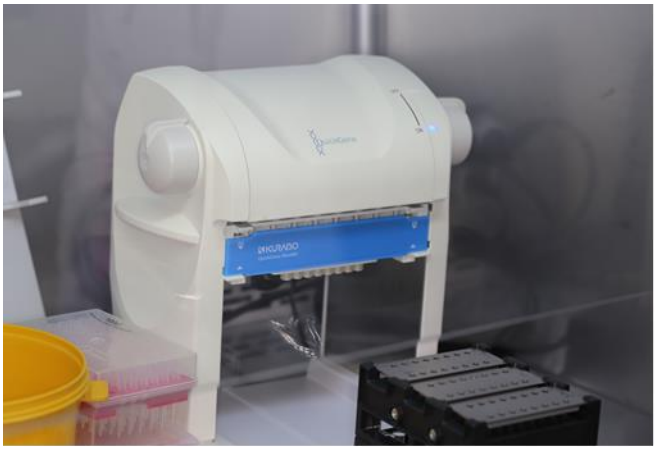
In order to precipitate and bind the DNA to a purification matrix, solution-based chemistry or exchange (silica, cellulose or ion exchange) methods are used. Once the DNA is precipitated, it is further washed using wash buffers such as ethanol, and then eluted to solubilize DNA in an elution buffer such as tris-EDTA or nuclease-free water to protect it from degradation.

4. Assessing DNA purity[40]

The quality of the isolated DNA sample needs to be assessed before further analysis. This can be performed using absorbance, electrophoresis and fluorescence methods. The most common method is absorbance analysis using a spectrophotometer and measuring absorbance from 230 nm to 320 nm. Since DNA absorbs light most strongly at 260nm (A_{260}), the most commonly used ratio of absorbance used during this analysis is A_{260}/A_{280} ratio. An A_{260}/A_{280} ratio of 1.7–2.0 indicates high-quality DNA with low amount of contaminants. After these steps are completed, the isolated high-quality DNA is ready to use in PCR analysis.

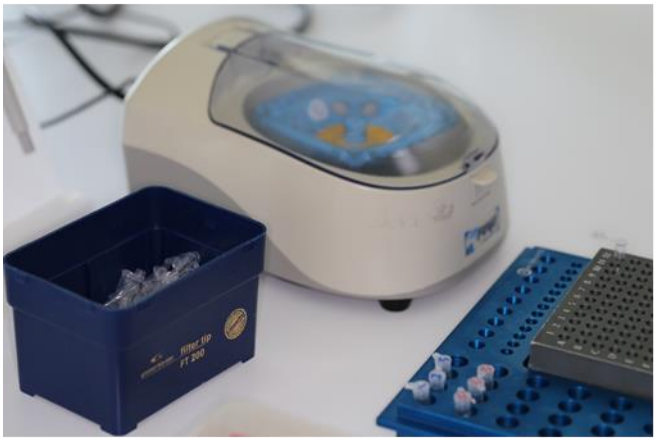
*Also see Figure 3.

Appendix V: Some laboratory devices used in experiments.



Automated nucleic acid isolation system

Homogenizer and dry bath incubator



Centrifuge, tubes and tube holders

Quantitative PCR cycler