EFFECT OF CAFFEINE ON HORIZONTAL GENE TRANSFER

A Thesis
by
QIUSHI LIANG

Submitted to the Graduate and Professional School of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Chair of Committee, Qing Sun
Committee Members, Hung-Jen Wu
Arum Han
Xuejun Zhu

Head of Department, Victor Ugaz

August 2022

Major Subject: Chemical Engineering

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ABSTRACT

Microbiomes have drawn a large interest in the scientific community in recent years (Hornung et al, 2019). Currently, it is postulated that Horizontal Gene Transfer (HGT), a common phenomenon that occurs across many bacterial species, happens in human gut microbiome as well (Liu et al, 2012). The flow of genetic information is fluid amongst this domain and can be exchanged between organisms through mechanisms such as transformation. Many studies have attributed that bacteria have co-evolved with their' environment to survive. Wang et al. 2020 proposed that an exterior stressor evoked bacteria's sense of survival which enhanced the DNA to uptake the recombination that ultimately promotes transformation (Wang et al 2020. In this case, shifting away from a normal growth environment has been suspected to induce stress to many different bacteria in many ways. With anti-bacterial effects, caffeine can successfully act as an exterior stressor to stimulate the transformation process. Through experiment, with exposure to caffeine in *Escherichia coli* culture, caffeine showed an enhancement effect on bacterial transformation. With preliminary RNA sequencing results, it was found that *fumC*, genetic sequence that encodes fumarase enzyme, were upregulated drastically when the sample is exposed to caffeine, along with its counterpart, *fumA* and *fumB* being deactivated or downregulated. This showed the presence of increase in ROS level in the cell culture when exposed to caffeine. With reference to a study of similar interest, increase in ROS level and exterior stress level, the cell permeability was increased, enhancing horizontal gene transfer. Therefore, it was hypothesized that with the presence of caffeine, the antimicrobial effect served

as an exterior stressor to the bacteria, and that the bacteria's responses to stress would increase DNA uptake and recombination, promoting the bacterial transformation.

DEDICATION

To my two families, one in China and the other in America: Xiao Liang, Hong Luo, Jianhong Li, and Jing Lin, who have always believed in me and delivered with full support throughout this journey. To my Principal Investigator, Dr. Qing Sun, for all the guidance and help along my master's degree. To my teammate, Tracy Mei, for all the mentorship and support provided in my master's project.

ACKNOWLEDGEMENTS

I would love to express my most sincere gratitude to my committee chair, Dr. Qing Sun, for her endless support and guidance in my master's degree, both emotional and professional. Dr. Sun is truly the best mentor, providing me with an abundance of resources and guidance, granting me an amazing opportunity to participate in projects pertaining to bioinformatics and genetics. Even though my background in Chemical Engineering has presented a tremendous amount of challenges throughout my research, with Dr. Sun's help, I slowly conquered those obstacles and adapted to the research.

I would like to thank my committee members, Dr. Hung-Jen Wu, Dr. Xuejun Zhu, and Dr. Han for supporting me and attending my thesis defense. I would like to thank Dr. Andrew Hillhouse and Wesley Brashear in Texas A&M institute for Genome Sciences and Society for the resources and support they convoyed regarding bioinformatics. Without their support, I would have never finished my master's research project.

Moreover, I would like to give my special appreciation to my friends and lab mates. I would specially like to thank my partner, Tracy Mei, for being patient and taking her time mentoring and working with me on designing experiments and developing research. Tracy has been an amazing colleague, mentor and friend to work with. The spirit of a true scientist resides in her through her diligence and rigorous yet supportive attitude in the research. I would also like to thank Dr Min Feng, Rushant Sabnis, Baizhen Gao, Siddhant Gulati, Siddhi Kotnis, and Shujun He from Dr. Sun's lab. It is because of them that my life in Texas A&M has been full of joy, and my experience and transition in the lab have been fulfilling. I would like to thank my roommates, BingQian Wu and Yiwei Mao, for accompanying me throughout this journey. I would love to thank friends I have encountered within my master's degree, including Sek-Kai Leong, Xuele

Lin, and Alex Bokenstedt, for their support and friendship walking alongside with me in my persuasion of master's degree.

I would like to thank Dr. Mustafa Akbulut for his amazing lecture on Thermodynamics and Dr. Victor Ugas for his excellent lecture on Microfluid Dynamics and Technology. Because of their informational and fantastic lectures, I was able to attain knowledge in semiconductor field, along with all the experience and techniques I improved and learned in Dr. Sun's lab, which helped me acquire my full-time position in a high-tech company.

Finally, I would love to thank my families, Xiao Liang, Hong Luo, Jianhong Li, and Jing Lin for their unconditional support and endless love throughout my graduate education. It is them that push me through this journey.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supported by a master's dissertation committee consisting of committee chair Dr. Qing Sun of the Department of Chemical Engineering and committee members Dr. Hung-Jen Wu, Dr. Xuejun Zhu of the Department of Chemical Engineering, as well as Dr. Arum Han of the Department of Electrical & Computer Engineering.

Funding Sources

This work was supported by Artie McFerrin Department of Chemical Engineering and Dr Qing Sun's laboratory startup funds.

ABBREVIATIONS

ARG	-	Antibiotic Resistance Gene
CNS	-	Central Nerve System
GRAS	-	Generally Recognized as Safe
HGT	-	Horizontal Gene Transfer
LB	-	Lysogeny Broth
OD	-	Optical Density
PBS	-	Phosphate Buffered Saline
ROS	-	Reactive Oxygen Species

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CHAPTER I

INTRODUCTION

With over 2.25 billion cups being consumed daily worldwide, coffee is one of the most popular beverages in the world(ref). One of the key components, 1,3,7-Trimethylxanthine (caffeine), amounts to an average of 90 mg per 8 fluid ounces of coffee, is one of the most consumed stimulants. Additionally, caffeine is also found in sodas, tea, and energy drinks that also have a high consumer count. The average American consumes anywhere from 110-400mg/day of caffeine. Caffeine has shown to have antimicrobial effects on the gut microflora of the host. Hedge et al in 2019 have shown that coffee consumption alters the composition of the natural flora in feces compared to the control (Shrilakshmi et al, 2019).

Furthermore, studies have shown that caffeine has antibacterial properties that suppress the growth of bacteria (von Wintersdorff et al, 2016). Recent studies have shown that caffeine exhibits inhibitory effect including compromising cell wall integrity of yeast and it is speculated that the caffeine has the same effect to prokaryotes as well (Whitney et al, 2015).

Nonetheless, it is found by studies that caffeine induces SOS response to prokaryotes, a cellular response to DNA damage where the cell cycle is inhibited and DNA repairment process is induced (Handel et al, 2015). Even though the detailed pathway of caffeine triggering SOS response to prokaryotes is not researched, one of major ways to induce SOS response is by abundance of reactive oxygen species (ROS) (Handel et al, 2015). Studies have also shown with

some presence of ROS, triggering SOS, serve as exterior stress can enhance the horizontal gene transfer (HGT) (Wang et al, 2020).

Research has shown that other than caffeine, some non-antibiotic pharmaceuticals such as ibuprofen and diclofenac also present abilities to increase stress levels by producing excessive ROS. The exterior stress, in this case, ROS, created by the presence of those non-antibiotic pharmaceuticals is found to enhance the bacterial transformation, and speculated to contribute to the spreading of antibiotic resistance (Wang et al, 2020).

Horizontal gen transfer (HGT) among bacteria is one of the most common phenomena for bacteria communication and survival. It is speculated that HGT in bacteria residing in gastrointestinal tract can be affected by the common chemical compound and foodstuff intake. Such HGT process might has direct impact on human health due to the nature and importance of gut microbiome. One of the most relevant examples is the spread of ARGs through HGT that changes the safety status of a strain that belongs to Generally Recognize as Safe (GRAS) through attaining the anti-biotic genes (ARGs). However, the extent of HGT affecting the microbiome that is related to food and common chemical compounds in the gastrointestinal tract has not been established (Chung et al, 2019).

Even though the main mechanism of HGT of plasmids is recognized to be conjugation, natural transformation is still considered one of the major mechanisms in the genetic exchange in microbes (Norman et al, 2009). However, it is still unclear how and why natural transformation takes place within the bacterial community. Therefore, in this study, the natural transformation affected by caffeine is evaluated.

In many studies, HGT has been discovered to be one of the major contributors to the spread of anti-biotic resistance genes (ARG) (Rossi et al, 2014). Especially in recent decades, because of the selective pressure from more and more frequent human usage of antibiotics, the rate and chance for the spread of ARGs by HGT are drastically increased. With three mechanisms of HGT, for a long time, conjugation was viewed as the most impactful HGT mechanism on such matter. However, many studies have showed that the effect of bacterial transformation on the spread of ARGs is largely underestimated (Rossi F et al, 2014).

In this study, the spread of ARGs through one of the major methods of HGT, bacterial transformation, with caffeine and potentially other food nanoparticles are evaluated. Meanwhile, the spread of ARG through bacterial transformation can also indicate how caffeine affect the bacteria's ability to uptake foreign plasmid. In this case, how bacterial transformation are affected when exposed to caffeine in comparison to control samples was studied. The affected pathways and genes when exposed to caffeine are investigated through next-gene RNA sequencing tools. Moreover, how two strains interact with each other when exposed to different antibiotics and caffeine was also evaluated.

CHAPTER II

BACKGROUND AND RELATED WORK

2.1 Caffeine

Caffeine is a highly consumed stimulant commonly found in tea, coffee, and cacao plants. It is consumed at varying concentrations worldwide to stimulate the brain and central nervous system (CNS) to help consumers stay alert and increase productivity. 85% of the United States population consumes at least one caffeinated beverage a day (Mitchell et al, 2014). With caffeine being one of the highest consumed compounds by humans, studies have shown that caffeine has antimicrobial effects that can alter the microbiome of the host (Shrilakshmi et al, 2019). However, the mechanism of how the change in environment influences HRT remains unknown. (Soucy et al, 2015).

2.2 Horizontal Gene Transfer

Horizontal Gene Transfer (HGT) is the transfer of genetic information among organisms, a biological process that attributes to the spread of antibiotic resistance, promoting pathogen evolution, and changes organism’s DNA expression gradually (Burmeister et al, 2015). There are three mechanisms for horizontal gene transfer: bacterial conjugation, and bacterial transduction, bacterial transformation.

2.3 Bacterial Conjugation

With direct contact, bacterial conjugation allows bacteria to transfer genetic information to other bacteria. There are two roles in this process: the donor bacterium and the recipient

bacterium. The donor carries the fertility factor, a DNA sequence that allows bacterium to produce a thin tube called pilus. Through pilus, the plasmid of interest will be transferred to the recipient bacterium (Burmeister et al, 2015).

2.4 Bacterial Transduction

With bacteriophage, the virus targeting specifically prokaryotes, transduction can be executed with virus passing down the genetic information from one bacterium to another. The bacteriophage infects one bacterium and make it host to produce more viruses. These viruses reproduce and assemble within the host cell and occasionally carry pieces of DNA from the host cells during assembly stage and then later when infecting other bacteria, the virus will transmit the carried plasmid to the host cell (Burmeister et al, 2015).

2.5 Bacterial Transformation

Transformation refers to the process in which the organism uptake the free genetic material in the environment presented. Such process is a very common practice among biology study for the bacteria to uptake the plasmid of interest. This process can facilitate the spread of antibiotic resistance to microbiome within human body and can also be harmful when pathogenic strains reside in human body uptake the antibiotic resistance gene (Burmeister et al, 2015). Compared to other horizontal gene transfer mechanisms, transformation involves directly the exterior genetic elements from the surroundings (Sun et al, 2018).

2.6 Gastrointestinal (GI) Microbiome

The gastrointestinal tract, or digestive tract, is the pathway in which the intake of food and disposal of solid waste constantly happen. This tract consists of mouth, pharynx, esophagus, stomach, small intestine, large intestine, and anus. With the digestive process, elements that

initially do not belong to human body enter and reside in this tract, interacting with each other. This creates gastrointestinal microbiome, where more than 100,000 trillion microorganisms participate in daily activities in the gastrointestinal tract (Sommer et al, 2013).

While gastrointestinal tract hosts the one of the largest microbiomes in human body, the microbiome community evolution and the spread of antibiotic resistant bacteria are getting more attention due to their effects on human health and safety (Lamps et al, 2019). Antibiotic resistance has been more and more difficult to deal(Huddleston et al, 2014).

2.7 Relation between HGT and GI Microbiome

Microbiomes have drawn a large interest in the scientific community in recent years (Hornung et al, 2019). Currently, it is postulated that HGT, a common phenomenon that occurs across many bacterial species, occurs in many human microbes (Liu et al, 2012). The flow of genetic information is fluid amongst this domain and can be exchanged between organisms through mechanisms such as transformation. Many studies have attributed that bacteria have co-evolved with their' environment to survive. Wang et al. 2020 proposed that an exterior stressor evoked bacteria's sense of survival which enhanced the DNA to uptake the recombination that ultimately promotes transformation (Wang et al 2020). In this case, shifting away from a normal growth environment has been suspected to induce stress to many different bacteria in many ways. With anti-bacterial effects, caffeine can successfully act as an exterior stressor to stimulate the transformation process.

2.8 Antibiotic Resistance

Aforementioned, the spread of antibiotic resistance has largely hindered the treatment of bacterial infections (Huddleston et al, 2013.). The rise of antibiotic resistant issues especially in

recent decades, as the abundant usage of antibiotics, is seen as a serious global individual health concern (Levy et al, 2014). It is thought that the antibiotic therapy's golden age is drawing to a close, and that individuals may soon regress to a pre-antibiotic era with few effective treatments for bacterial infections. These resistant infections are costly to society in terms of both economic effect and morbidity and mortality among those affected. Each year, thousands of people die from methicillin-resistant Staphylococcus aureus and third-generation cephalosporin-resistant infections, resulting in thousands of infections and millions of dollars spent on health care costs (de Kraker et al, 2011). Antibiotic development by pharmaceutical corporations has reduced, despite the fact that infectious diseases are still the biggest cause of mortality worldwide (Armstrong et al, 1999; Spellberg et al, 2004). Adaptations of antibiotic resistance proceeds, alongside strains that have multidrug resistance, blockading the bacteria infection treatments (Levy et al, 2014).

2.9 Relation among HGT, GI tract, and Antibiotic Resistance

Bacterial populations that are exposed to antibiotics can adapt the antibiotic resistance by genetic mutation or horizontal gene transfer to gain the expression of resistance genes from other strains, regardless of whether it is far related or closely related. Antibiotic resistance genes can emerge and spread in bacterial populations thanks to the ideal conditions created by the human gastrointestinal tract. For instance, there is a high cell density and a high abundance of free genetic elements existing in the GI tract (Huddleston et al, 2013).

While the main mechanism in HGT contributing to the transfer of plasmid within GI microbiome is conjugation, the natural transformation is also considered as the major method of plasmid transfer (Norman et al, 2009).

CHAPTER III

MATERIAL AND METHOD

2.10 Bacteria and Cell-free plasmid

The naturally competent *Escherichia coli* (*E. coli*) NEB5α and *E. coli* BW25113 were used in this study. The plasmid, pET-21a, which encodes resistance to carbenicillin, was extracted from *E. coli* NEB5α and *E. coli* BW25113 respectively for different experiments, and pET-24a, which encodes resistance to kanamycin, was extracted from *E. coli* BW25113. All the plasmid extraction were done by Quantum Prep® Plamsmid Midiprep kit (Bio-rad, USA) and used as the exogenous gene transfer agent. The optical density (OD) of the sample was obtained through microplate spectrophotometer Epoch 2 (Agilent, USA).

2.11 RNA Sequencing

RNA sequencing (RNA-Seq) utilizes the ability of high-throughput sequencing methods to give a thorough insight on the transcriptome of a eukaryotes or prokaryotes cell. With high-power computer, RNA-seq provides a much higher coverage on the transcriptome of a cell than previous methods (Kukurba et al, 2014). RNA sequencing consisted of several part: RNA extraction, reverse transcription into cDNA, adapted ligation, amplification, and sequencing. In this case, the RNA sequencing was executed in the RNA sequencing facility within Texas A&M.

In this work, 3 sets of samples from experiment: bacterial transformation exposed/without exposed to caffeine, which will be covered in the next section, were sent to sequencing facility located in Texas A&M Institute for Genome Sciences and Society for rRNA extraction and RNA sequencing with the assistance of Wesley Brashear and Dr. Andrew Hillhouse. After sequencing,

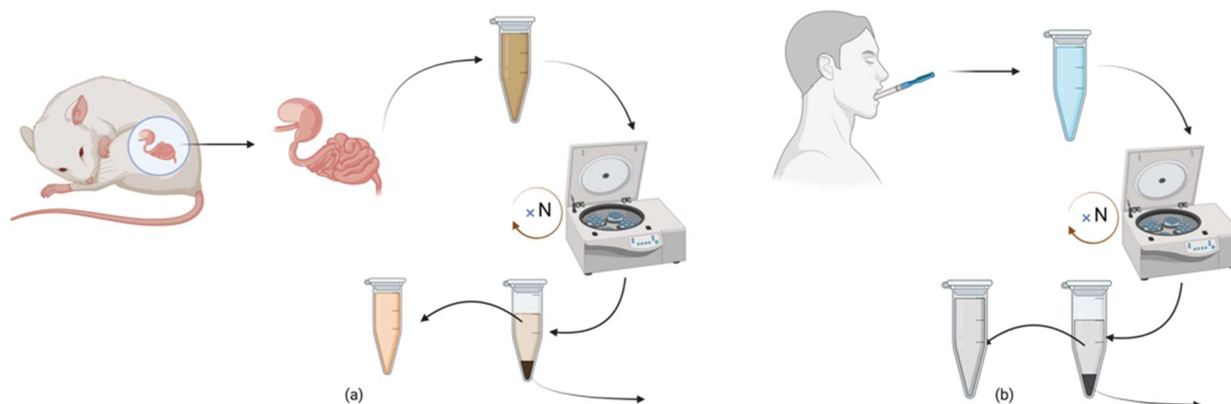

Figure 1. Substrate Preparation: a) gut fluid extraction and purification and b) saliva extraction and purification

the raw data was sent back in the file format of *.gff* for downstream analysis. The analysis will be covered in the result and discussion session.

2.12 Experiment and Procedure

The following section covers the experiment and procedure involved in this project: caffeine exposed bacterial transformation and cell to cell transformation in liquid culture

2.13 Bacterial Transformation Exposed to Caffeine

To evaluate the bacterial transformation in GI microbiome, both gut fluid and saliva were collected and disinfected as the environment buffer. As depicted in figure 1.a, The entire gastrointestinal tract was taken out from mice with both ends saw to seal. The gut fluid was then extracted into tubes and then centrifuged at 15000 rpm for 10 min. Supernatant was taken out to a new tube, resuspended, and centrifuged again. Centrifugation repeats until there is no precipitation in the tube. The purified gut fluid is then collected for further evaluation. Similarly,

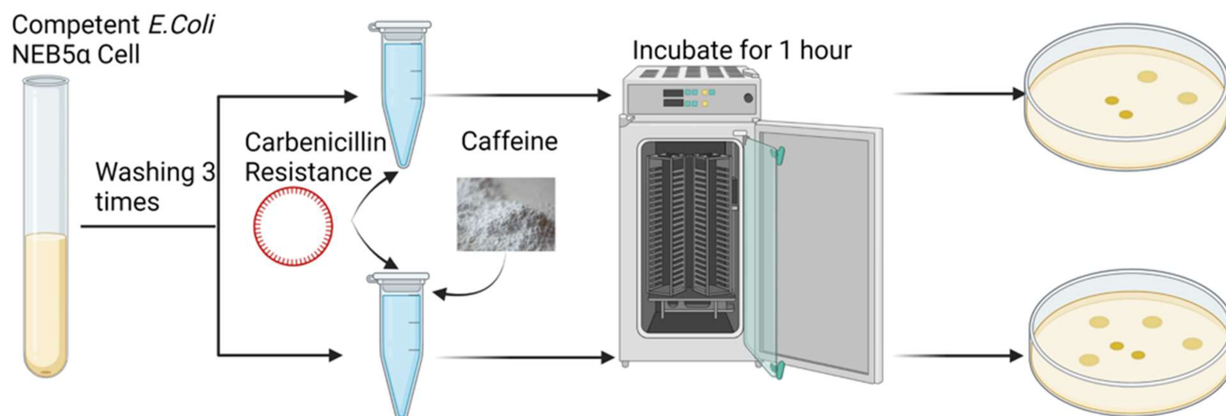

Figure 2. Caffeine Exposed Bacterial Transformation

as depicted in figure 1.b, The saliva was donated from a volunteer and collected to tubes and then centrifuged at 15000 rpm for 10 min. Supernatant was taken out to a new tube, resuspended and centrifuged again. Centrifugation repeats until there is no precipitation in the tube. The purified saliva is then stored at -80° for further evaluation. However, the bacteria developed in laboratory has already adapted to the safe environment created in the lab and cannot survive in the hostile environment created by either gut fluid and saliva. Because this work is a proof of concept, it was decided that the substrate used in this work would be phosphate buffer solution (PBS).

E. coli NEB5α has an innate ability to acquire and spread exogenous DNA, with it, one examined its capabilities for HGT under the presence of caffeine (Chen et al, 2019; Hasegawa et al, 2018; Riva et al, 2020). For caffeine exposed bacterial transformation, as depicted in figure 2, a single colony of *E. coli* NEB5α was taken from a solid lysogeny broth (LB)- agar plate and incubated overnight in 3 mL of LB broth at 37°Cwith XXX spead shaking at 250 rpm. Then, 60 mL of overnight culture was taken out and diluted into 6 mL of LB broth, put into an incubator at temperature of 37°C and shaking speed of XXX. The diluted sample is incubated until OD of 0.6, which represents the beginning of the exponential phase of *E. coli* NEB5α for competency.

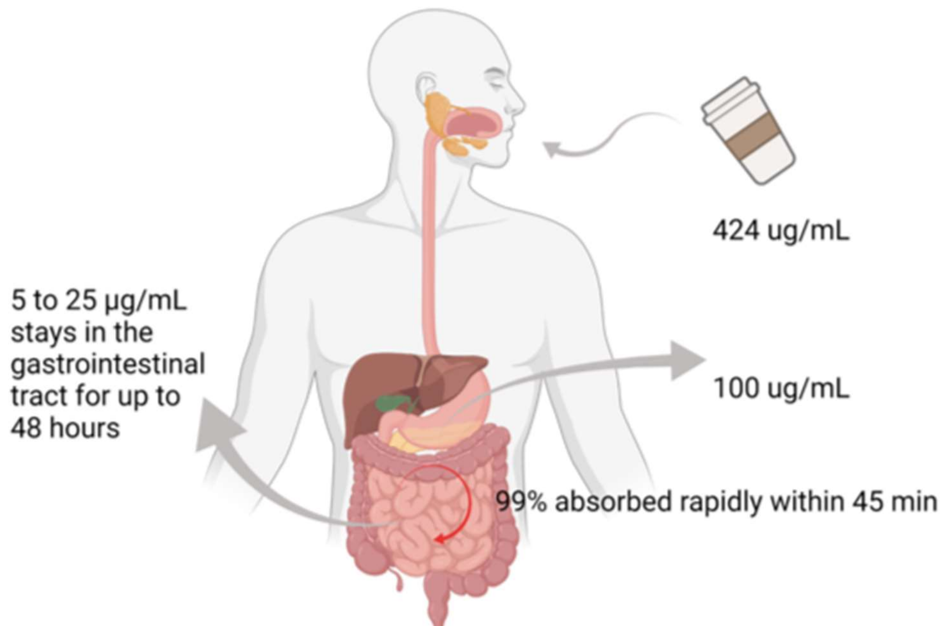

Figure 3. Caffeine Concentration Identification

This process took approximately 3.5 hours in total. After the bacteria reached competency, 4 samples of approximately $3.5*10^7$ cells were taken out and stored into 1.5 mL tubes. The volume was calculated by equation (1):

$$V = \frac{N}{2.66 \times 10^9 * OD} \tag{1}$$

As V represents the volume needed to be taken out of the competent sample in the unit of milliliter, OD represents the OD reading from the microplate spectrophotometer, and N represents the number of cells needed by the experiment. In this case, an OD of 1 represented 2.66×10^9 cells per millimeter in the sample. After a certain volume was taken out for each sample, the samples were centrifuged down at a speed of 8000 rpm for 3 min to collect the cells. After removing the supernatant, each sample was resuspended in1 mL PBS. The samples were resuspended in PBS and washed two more times with PBS. With most of the LB washed off by

PBS solution, the bacteria sample would not have any lateral gene transformation since there is no nutrient in the sample for bacteria to propagate. Therefore, solely the bacterial transformation would be observed in this experiment. After centrifugation, the supernatant was taken out and the sample was resuspended with another 1 ml of PBS solution. The washing step was repeated three times to make sure most of the LB was taken out of the sample. At the end of the washing step, the bacterial samples were resuspended into 100 µl of PBS solution, waiting for the final step of sample preparation.

The samples were split into two groups. With 1 sample in the control group, the other 3 samples were in the caffeinated group. As implied by the name, the control sample would not contain any caffeine, yet the other 3 samples would have caffeine added with respective concentrations. With caffeine added, the 3 samples each contained 5 µg/ml, 10 µg/ml, 15 µg/ml, and 25 µg/ml caffeine respectively. Those concentrations were chosen according to figure 3 using 1 cup of coffee as an example, the caffeine remaining in the gastrointestinal tract up to 48 hours was approximately 5 µg/ml to 25 µg/ml. At the end, around 2000 plasmids were added to each sample. The samples were then put into an incubator for incubation for 3 hours at a constant temperature of 37 °C and shaken. After 3-hour incubation, the samples were taken out and plated on a LB-Agar solid plate containing working concentrations, 1µg/ml, of carbenicillin antibiotics. Colonies were counted the second day for all the samples and data was recorded for analysis.

3.1.1 Cell to Cell Transformation Exposed to Caffeine in Liquid Culture

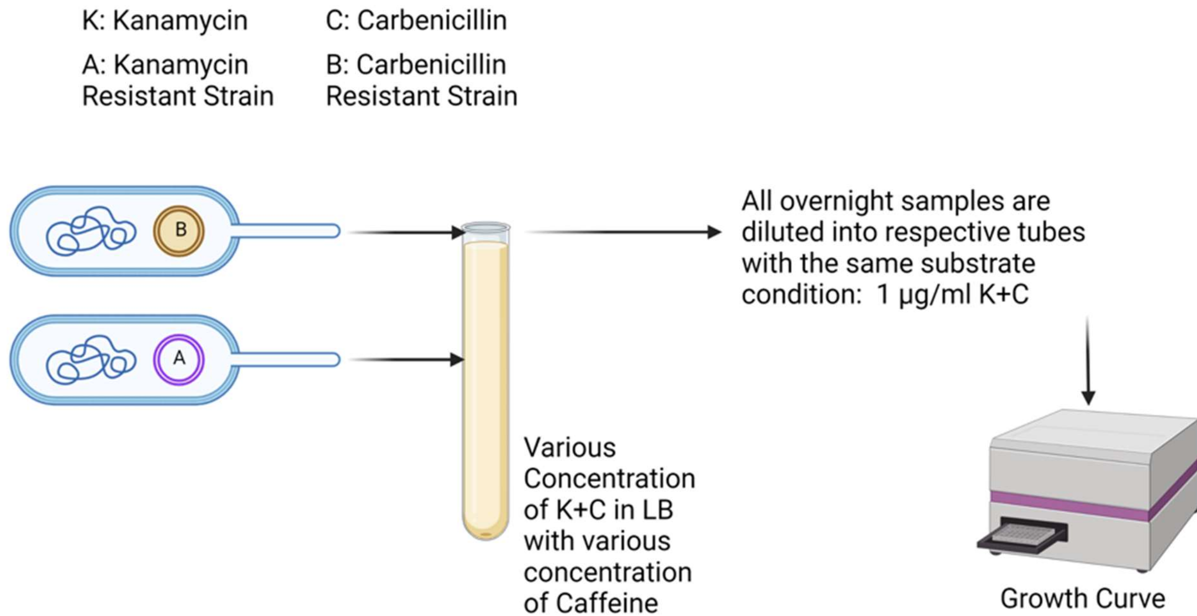

Figure 4. Cell to Cell Transformation in Liquid Culture

To study the effect of caffeine on the interaction between cells, caffeine exposed cell to cell transformation in liquid culture was designed. As depicted in figure 4, two samples of *E. coli* BW25113, one contains kanamycin resistant plasmid and the other contains carbenicillin resistant plasmid, were incubated overnight at a constant temperature of 37 °C and shaken. After the first overnight incubation, two samples were diluted into 15 samples. Utilizing equation 1, each sample contains $1*10^8$ bacteria from both bacterial culture and various other conditions shown in table 1. Those samples were then incubated overnight at a constant temperature of 37 °C and shaken. After overnight incubation, OD of all samples were obtained and diluted into 200 µl LB with 3 technical replicates and starting OD of 0.01, each stored in one well of a 96 well plate. The plate was then put into microplate spectrophotometer for over 10 hours at a contant temperature of 37 °C and shaken to develop a growth curve for each sample.

Table 1. Sample number and its conditions for cell to cell transformation

Sample #	K+C concentration (µg/ml)	Caffeine Concentration (µg/ml)
1	0	0
2	0.5	0
3	1	0
4	0	5
5	0.5	5
6	1	5
7	0	15
8	0.5	15
9	1	15
10	0	25
11	0.5	25
12	1	25
13	0	50
14	0.5	50
15	1	50

CHAPTER IV

RESULTS AND DISCUSSION

2.14 Caffeine Exposed Bacterial Transformation

The colony forming unit (cfu) was counted from the caffeinated exposed bacterial transformation experiment as shown in figure 5. The x axis of this graph represents the various concentration of caffeine existed in the sample, and the y axis represents the average cfu count after plating the incubated samples to a solid LB-Agar plate.

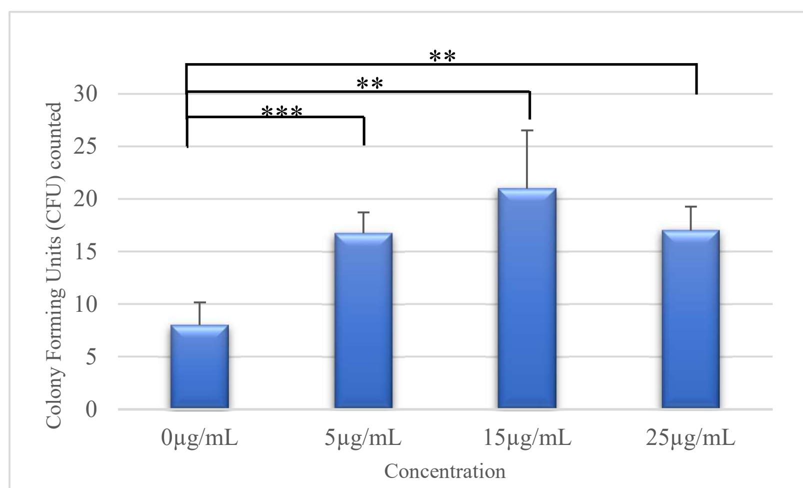

Figure 5. Results of Caffeinated Exposed Bacterial Transformation

As one can see, caffeine has enhancement effect on bacterial transformation. At 0 μg/mL of caffeine, with 2000 plasmid added, the overnight solid LB-Agar plate had averagely 8 colonies. At 5 μg/mL of caffeine within the sample, with the same amount of plasmid added, the overnight solid LB-Agar plate had averagely 16.75 colonies. At 15 μg/mL of caffeine within the sample, with the same amount of plasmid added, the overnight solid LB-Agar plate had averagely 21 colonies. Finally, at 25 μg/mL of caffeine within the sample, with the same amount of plasmid added, the overnight solid LB-Agar plate had averagely 17 colonies.

One tailed paired T test was conducted on each sample in comparison with the control, the sample with 0µg/mL of caffeine. The results showed statistical significance in terms of p-value. Between sample with 0 µg/mL and 5 µg/mL of caffeine, the p-value was calculated to be 0.00068. Between sample with 0 µg/mL and 15 µg/mL of caffeine, the p-value was calculated to be 0.04109. Between sample with 0 µg/mL and 25 µg/mL of caffeine, the p-value was calculated to be 0.01807. As one can see, different concentrations of caffeine delivered different amount of enhancement on bacterial transformation. Among all the samples, the sample with 15 µg/mL showed the most promising results. Therefore, it was determined that when moving forward to RNA sequencing, one set of samples will be consisted of one control, with 0 µg/mL of caffeine in the sample, and one caffeinated sample, with 15 µg/mL of caffeine in the sample.

In conclusion, caffeine appeared to have an enhancement effect on bacterial transformation. Among all the samples, sample with caffeine concentration of 15 µg/mL showed the most efficient result.

2.15 RNA-Sequencing Results

Before sending the sample to RNA sequencing facility, caffeinated exposed bacterial transformation experiment was conducted with 3 biological replicates. After confirming that the results of the experiment aligned with collected data, the 3 sets of samples were then delivered to RNA-sequencing facility for sequencing.

In order to further evaluate the potential mechanisms involved in the enhanced transformation, the gene and protein expression levels between control sample and caffeine exposed sample were compared. This process was completed with measuring protein abundances

and genome-wide RNA sequencing. With addition of caffeine to the sample, genes relating to ROS and SOS response were found to be either upregulated or downregulated.

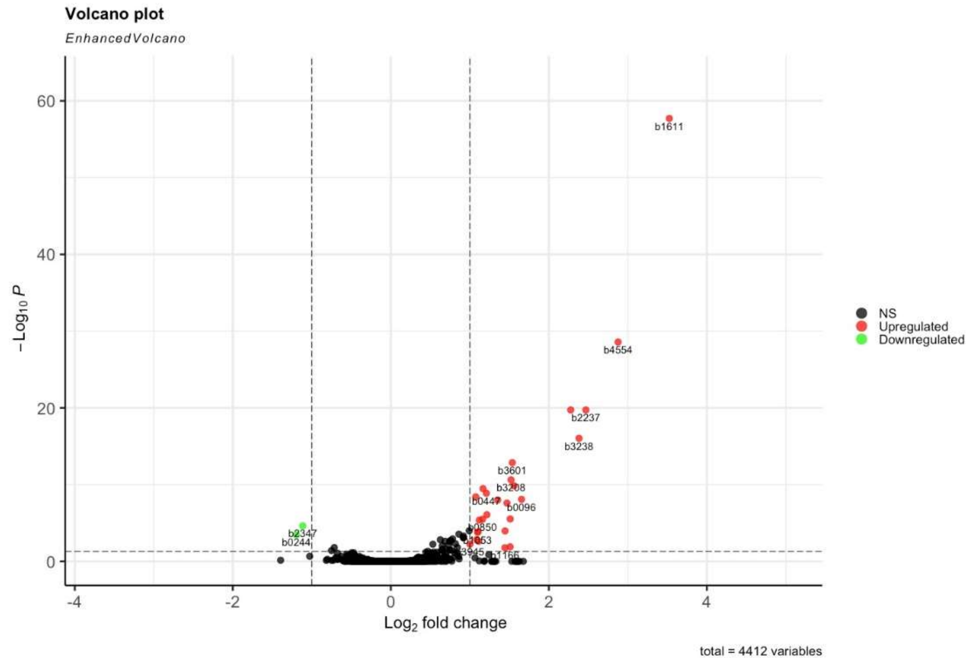

Figure 6. Volcano Plot of the RNA sequencing Results

As depicted in figure 6, the result of RNA sequencing is expressed in the form a volcano plot, where x axis represents $\log_2$ fold change and y axis represent $\log_{10}$ value of p-value. That is, the greater x value a data point has represented higher magnitude change and the greater y value a data point has represented higher statistical significance. Among many genes that has been upregulated, gene ID b1611 showed upregulation in both high magnitude and statistical significance. This gene represented *fumC*, a genetic sequence encoding the functionality of producing fumarase enzymes of oxidative tricarboxylic acid (TCA) cycle. Genes that serve similar functionality as *fumC* are *fumA* and *fumB*, which in this case, showed downregulation in RNA sequencing result. In this case, fumC is upregulated approximately 3.5 2-fold changes in magnitude with a confidence level higher than 99.99%.

2.16 Relationship between ROS and fumC.

It is found by studies that highly oxidized condition inactivated *fumA* and *fumB* activities as the oxygen level rises, but *fumC* activity increased simultaneously when the oxygen level was high (Tseng et al, 2001). Tseng, C. P. et al has tested multiple strains of E. coli, and fumarases were regulated in the same way in response to varying oxygen levels. As a result, the three fumarase enzymes' s synthesis is controlled in a hierarchical manner based on the amount of oxygen available in the environment (Tseng et al, 2001). High oxygen level usually directly links to high presence of ROS in the cell, as oxidative stress is increased by the increase of oxygen. Furthermore, studies have found that superoxide radicals, which is a common ROS, also causes increased *fumC* gene expression (Park et al, 1995).

Such phenomenon was also found in this work. With caffeine presented in the sample, cell underwent a drastic upregulation in the gene expression of *fumC*, along with downregulation in *fumA* and *fumB*. With upregulation of fumC and deactivation of fumA and fumB, the result showed that ROS level has increased to an extend within the sample containing caffeine.

ROS are frequently encountered by Escherichia coli in its surroundings, most commonly in the form of H2O2 produced as a consequence of other gut microorganisms' metabolic activity, such as lactic acid bacteria (Mendoza-Chamizo et al, 2018). Aforementioned, ROS such as superoxide radicals was determined to be raised in the sample exposed to caffeine, and HO• is a highly reactive oxidant that reacts with organic molecules in its immediate proximity. HO• causes DNA damage when H_2O_2 interacts with iron bound to DNA (Cadet et al, 2013). In this case, DNA damage would trigger SOS response, a global response of prokaryotes to DNA damage in which the cell-division cycle was inhibited and DNA repair and mutagenesis were upregulated. Therefore, with *fumC* upregulated, ROS was present in the sample when exposed

to caffeine, leading to SOS response. Such response represents the existence of exterior stress that affected cell's survivability.

As a result of the presence of exterior stressors, Bacteria may produce more ROS, stress responses may be triggered, and cell membranes may change (Brynildsen et al, 2013; Hong et al, 2019). It was hypothesized that with the presence of caffeine, the antimicrobial effect served as an exterior stressor to the bacteria, and that the bacteria's responses to stress would increase DNA uptake and recombination, promoting the transformation effect.

Studies of similar interest have done examinations to verify these hypotheses by utilizing fluorescence techniques and cell membrane permeability dye peptide iodine to measure the ROS production and cell membrane permeability in the presence of varied quantities of non-antibiotic medicines. Those non-antibiotic medications such as ibuprofen and naproxen also have the ability to affect the bacteria to over produce ROS, similar to caffeine, and enhance the bacterial transformation (Wang et al, 2020). It was found that with increasing ROS level, the cell permeability was increased, and the bacterial transformation was enhanced, contributing to the spread of ARGs.

Similar hypothesis was proposed in this work: caffeine served as exterior stress that promotes the production of ROS and increases the cell permeability, which ultimately enhanced the bacteria's ability to uptake foreign genetic elements in the environment.

2.17 Cell to Cell Transformation

After the procedures depicted in figure 4, the samples were taken into a microplate reader for growth curve development. However, the result showed that this experiment needed further optimization, as the samples failed to grow in the culture containing working concentration of both antibiotics.

The overnight sample containing various concentration of caffeine and antibiotics, conditions showed in table 1, were transferred into a 96 well microplates. Each well contained the same amount of cells from one of 15 samples and filled with PBS solution up to 200 µL. At the end, each well would contain 3×10^6 cells, which represents the optical density of 0.01; each well also would contain working concentration of antibiotics, 1 µg/mL. Each sample was put into respective wells of the 96-well plate technical triplicates.

However, the cells from all samples did not grow in the plate, as the growth curve reached the stagnant phase immediately within 15 to 30 mins of development, and the there was no growth shown in the curve at all. Therefore, the experiment still needed to be optimized further. It was suspected that the survival rate was too low when the cells were exposed to antibiotics and carrying two strains at the same time creates burdens to the bacteria. Therefore, it was hypothesized that the cell could survive and transform when put into a larger volume of medium with the same optical density, meaning more cells to start from compared to only 200 µL medium when developing growth curve in a 96-well plate. With larger starting cell number, the rate of survival could be improved.

CHAPTER V

SUMMARY AND CONCLUSIONS

In this study, the spread of ARGs through one of the major methods of HGT, bacterial transformation, with caffeine and potentially other food nanoparticles are evaluated. Meanwhile, the spread of ARG through bacterial transformation can also indicate how caffeine affect the bacteria's ability to uptake foreign plasmid. In this case, how bacterial transformation are affected when exposed to caffeine in comparison to control samples was studied. The affected pathways and genes when exposed to caffeine are investigated through next-gene RNA sequencing tools. Moreover, how two strains interact with each other when exposed to different antibiotics and caffeine was also evaluated.

Through experiment, with exposure to caffeine in the Escherichia coli culture, the caffeine showed an enhancement effect on bacterial transformation. With RNA sequencing, it was found that *fumC*, genetic sequence that encodes fumarase enzyme, were upregulated drastically when the sample is exposed to caffeine, along with its counterpart, *fumA* and *fumB* being deactivated or downregulated. This showed the presence of increase in ROS level in the cell culture when exposed to caffeine. With reference to a study of similar interest, increase in ROS level and exterior stress level, the cell permeability was increased, enhancing horizontal gene transfer. Therefore, it was hypothesized that with the presence of caffeine, the antimicrobial effect served as an exterior stressor to the bacteria, and that the bacteria's responses to stress would increase DNA uptake and recombination, promoting the bacterial transformation.

LITERATURE CITED

Armstrong, G. L., Conn, L. A., & Pinner, R. W. (1999). Trends in infectious disease mortality in the United States during the 20th century. *JAMA*, *281*(1), 61–66. https://doi.org/10.1001/jama.281.1.61

Brynildsen, M. P., Winkler, J. A., Spina, C. S., MacDonald, I. C., & Collins, J. J. (2013). Potentiating antibacterial activity by predictably enhancing endogenous microbial ROS production. *Nature biotechnology*, *31*(2), 160–165. https://doi.org/10.1038/nbt.2458

Burmeister A. R. (2015). Horizontal Gene Transfer. *Evolution, medicine, and public health*, *2015*(1), 193–194. https://doi.org/10.1093/emph/eov018

Cadet, J., & Wagner, J. R. (2013). DNA base damage by reactive oxygen species, oxidizing agents, and UV radiation. *Cold Spring Harbor perspectives in biology*, *5*(2), a012559. https://doi.org/10.1101/cshperspect.a012559

Chen, Q. L., Cui, H. L., Su, J. Q., Penuelas, J., & Zhu, Y. G. (2019). Antibiotic Resistomes in Plant Microbiomes. *Trends in plant science*, *24*(6), 530–541. https://doi.org/10.1016/j.tplants.2019.02.010

Chung W. H. (2021). Pleiotropic Effects of Caffeine Leading to Chromosome Instability and Cytotoxicity in Eukaryotic Microorganisms. *Journal of microbiology and biotechnology*, *31*(2), 171–180. https://doi.org/10.4014/jmb.2011.11042

de Kraker, M. E., Davey, P. G., Grundmann, H., & BURDEN study group (2011). Mortality and hospital stay associated with resistant Staphylococcus aureus and Escherichia coli bacteremia:

estimating the burden of antibiotic resistance in Europe. *PLoS medicine*, *8*(10), e1001104. https://doi.org/10.1371/journal.pmed.1001104

Händel, N., Hoeksema, M., Freijo Mata, M., Brul, S., & ter Kuile, B. H. (2015). Effects of Stress, Reactive Oxygen Species, and the SOS Response on De Novo Acquisition of Antibiotic Resistance in Escherichia coli. *Antimicrobial agents and chemotherapy*, *60*(3), 1319–1327. https://doi.org/10.1128/AAC.02684-15

Hasegawa, H., Suzuki, E., & Maeda, S. (2018). Horizontal Plasmid Transfer by Transformation in *Escherichia coli*: Environmental Factors and Possible Mechanisms. *Frontiers in microbiology*, *9*, 2365. https://doi.org/10.3389/fmicb.2018.02365

Hong, Y., Zeng, J., Wang, X., Drlica, K., & Zhao, X. (2019). Post-stress bacterial cell death mediated by reactive oxygen species. *Proceedings of the National Academy of Sciences of the United States of America*, *116*(20), 10064–10071. https://doi.org/10.1073/pnas.1901730116

Hornung, B., Zwittink, R. D., & Kuijper, E. J. (2019). Issues and current standards of controls in microbiome research. *FEMS microbiology ecology*, *95*(5), fiz045. https://doi.org/10.1093/femsec/fiz045

Huddleston J. R. (2014). Horizontal gene transfer in the human gastrointestinal tract: potential spread of antibiotic resistance genes. *Infection and drug resistance*, *7*, 167–176. https://doi.org/10.2147/IDR.S48820

Kukurba, K. R., & Montgomery, S. B. (2015). RNA Sequencing and Analysis. *Cold Spring Harbor protocols*, *2015*(11), 951–969. https://doi.org/10.1101/pdb.top084970

LAMPS L. W. (2009). Infectious Disorders of the GI Tract. *Surgical Pathology of the GI Tract, Liver, Biliary Tract, and Pancreas*, 51–79. https://doi.org/10.1016/B978-141604059-0.50007-2

Levy, S. B., & Marshall, B. (2004). Antibacterial resistance worldwide: causes, challenges and responses. *Nature medicine*, *10*(12 Suppl), S122–S129. https://doi.org/10.1038/nm1145

Liu, L., Chen, X., Skogerbø, G., Zhang, P., Chen, R., He, S., & Huang, D. W. (2012). The human microbiome: a hot spot of microbial horizontal gene transfer. *Genomics*, *100*(5), 265–270. https://doi.org/10.1016/j.ygeno.2012.07.012

Mendoza-Chamizo, B., Løbner-Olesen, A., & Charbon, G. (2018). Coping with Reactive Oxygen Species to Ensure Genome Stability in *Escherichia coli*. *Genes*, *9*(11), 565. https://doi.org/10.3390/genes9110565

Mitchell, D. C., Knight, C. A., Hockenberry, J., Teplansky, R., & Hartman, T. J. (2014). Beverage caffeine intakes in the U.S. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*, *63*, 136–142. https://doi.org/10.1016/j.fct.2013.10.042

Norman, A., Hansen, L. H., & Sørensen, S. J. (2009). Conjugative plasmids: vessels of the communal gene pool. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, *364*(1527), 2275–2289. https://doi.org/10.1098/rstb.2009.0037

Park, S. J., & Gunsalus, R. P. (1995). Oxygen, iron, carbon, and superoxide control of the fumarase fumA and fumC genes of Escherichia coli: role of the arcA, fnr, and soxR gene products. *Journal of bacteriology*, *177*(21), 6255–6262. https://doi.org/10.1128/jb.177.21.6255-6262.1995

Riva, F., Riva, V., Eckert, E. M., Colinas, N., Di Cesare, A., Borin, S., Mapelli, F., & Crotti, E. (2020). An Environmental *Escherichia coli* Strain Is Naturally Competent to Acquire Exogenous DNA. *Frontiers in microbiology*, *11*, 574301. https://doi.org/10.3389/fmicb.2020.574301

Rossi, F., Rizzotti, L., Felis, G. E., & Torriani, S. (2014). Horizontal gene transfer among microorganisms in food: current knowledge and future perspectives. *Food microbiology*, *42*, 232–243. https://doi.org/10.1016/j.fm.2014.04.004

Shrilakshmi H., Daniel S., You-Min L., Xuan-Zheng P. S. (2019). Su1625 – In Vivo and in Vitro Effects of Coffee on Gut Microbiota and Smooth Muscle Contractility in Rats, *Gastroenterology*; 156:6, Supplement 1, Page S-587, ISSN 0016-5085. https://doi.org/10.1016/S0016-5085(19)38364-7

Sommer, F., & Bäckhed, F. (2013). The gut microbiota--masters of host development and physiology. *Nature reviews. Microbiology*, *11*(4), 227–238. https://doi.org/10.1038/nrmicro2974

Soucy, S. M., Huang, J., & Gogarten, J. P. (2015). Horizontal gene transfer: building the web of life. *Nature reviews. Genetics*, *16*(8), 472–482. https://doi.org/10.1038/nrg3962

Spellberg, B., Powers, J. H., Brass, E. P., Miller, L. G., & Edwards, J. E., Jr (2004). Trends in antimicrobial drug development: implications for the future. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, *38*(9), 1279–1286. https://doi.org/10.1086/420937

Sun D. (2018). Pull in and Push Out: Mechanisms of Horizontal Gene Transfer in Bacteria. *Frontiers in microbiology*, *9*, 2154. https://doi.org/10.3389/fmicb.2018.02154

Tseng, C. P., Yu, C. C., Lin, H. H., Chang, C. Y., & Kuo, J. T. (2001). Oxygen- and growth rate-dependent regulation of Escherichia coli fumarase (FumA, FumB, and FumC) activity. *Journal of bacteriology*, *183*(2), 461–467. https://doi.org/10.1128/JB.183.2.461-467.2001

von Wintersdorff, C. J., Penders, J., van Niekerk, J. M., Mills, N. D., Majumder, S., van Alphen, L. B., Savelkoul, P. H., & Wolffs, P. F. (2016). Dissemination of Antimicrobial Resistance in Microbial Ecosystems through Horizontal Gene Transfer. *Frontiers in microbiology*, *7*, 173. https://doi.org/10.3389/fmicb.2016.00173

Wang, Y., Lu, J., Engelstädter, J., Zhang, S., Ding, P., Mao, L., Yuan, Z., Bond, P. L., & Guo, J. (2020). Non-antibiotic pharmaceuticals enhance the transmission of exogenous antibiotic resistance genes through bacterial transformation. *The ISME journal*, *14*(8), 2179–2196. https://doi.org/10.1038/s41396-020-0679-2

Whitney, A. K., & Weir, T. L. (2015). Interaction of caffeine with the SOS response pathway in Escherichia coli. *Gut pathogens*, *7*, 21. https://doi.org/10.1186/s13099-015-0069-x

APPENDIX A

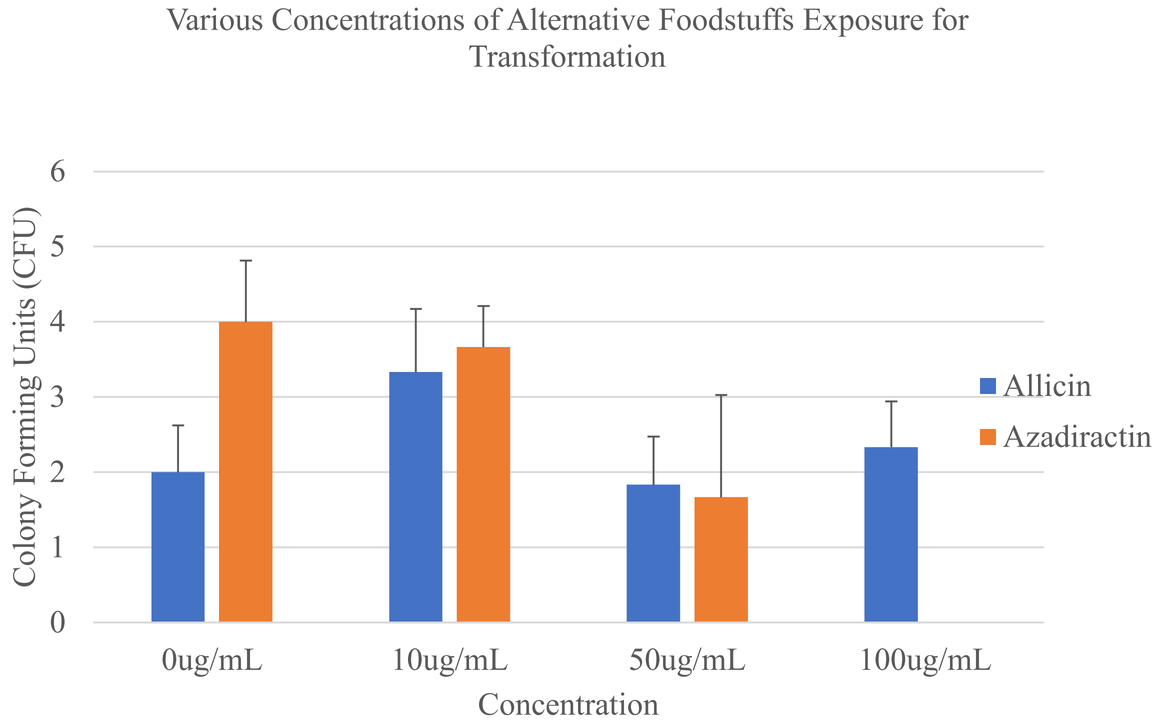

Figure 7. Attempting alternative food nanoparticles to test for similar results compared to caffeine. Allicin (blue) has no significant difference compared to control (n=6, p<0.15). Azadiractin (red) shows no significant difference compared to control (n=3, p<0.

Table 2. Differentially Expressed RNA Sequencing Data

	Base Mean	$Log_2FoldChange$	P_value	Adjusted P_value
b0003	7603.694	0.469207	0.000616	0.048305
b0005	344.4052	0.650992	0.000387	0.033509
b0096	187541.1	1.654069	2.36E-11	8.16E-09
b0127	1347.116	0.626065	1.11E-05	0.001544
b0244	11252.33	-1.19512	2.03E-06	0.000337
b0369	1848.034	0.781603	8.03E-06	0.001151
b0387	956.2797	0.766245	0.000434	0.036054
b0447	3871.576	1.209021	3.18E-12	1.32E-09
b0618	184.236	1.351358	3.17E-11	1.01E-08
b0850	652.1766	1.160182	1.35E-08	3.31E-06
b0851	1384.461	1.166719	7.28E-13	3.36E-10
b1036	887.4308	0.919265	5.51E-06	0.000839
b1053	988.6391	1.097597	8.80E-07	0.000159

b1166	5592.172	1.443416	0.000182	0.016769
b1167	4243.45	1.50995	0.000127	0.01261
b1463	2317.319	0.917472	5.65E-06	0.000839
b1530	187.3504	0.743642	0.000401	0.034011
b1531	297.4901	0.861522	1.65E-06	0.000286
b1611	25701.12	3.525101	4.65E-62	1.93E-58
b1612	32622.65	0.758794	1.49E-05	0.001933
b1833	5358.831	1.113416	2.09E-05	0.002411
b1851	4585.584	0.64215	0.000453	0.036902
b2143	9161.321	1.11987	1.84E-08	4.26E-06
b2159	2054.374	1.074192	1.05E-11	3.97E-09
b2201	1470.766	0.752563	1.61E-05	0.002021
b2202	734.034	0.817939	4.46E-05	0.005004
b2237	5900.754	2.468561	1.49E-23	1.85E-20
b2347	2197.795	-1.11427	1.06E-07	2.32E-05
b2390	3586.611	0.842282	0.000164	0.015688
b2493	1123.052	0.735026	6.71E-05	0.006972

b2801	1309.821	0.68904	0.000282	0.025464
b2924	4106.641	-0.75178	0.000493	0.039428
b2946	1797.668	0.532135	5.47E-05	0.005832
b3048	1066.43	1.470215	8.44E-11	2.51E-08
b3049	26118.31	2.27699	1.78E-23	1.85E-20
b3207	232442.2	1.558628	2.80E-13	1.45E-10
b3208	4534.782	1.521177	3.97E-14	2.36E-11
b3238	3162.17	2.381641	1.10E-19	9.16E-17
b3441	898.3184	1.105215	7.38E-07	0.000139
b3601	22088.1	1.537552	1.99E-16	1.38E-13
b3800	359.4623	0.747451	1.91E-05	0.002334
b3898	170.3775	0.991881	4.92E-07	0.000102
b3945	3039.134	1.001262	5.16E-05	0.005641
b4039	5045.289	1.215301	3.03E-09	8.39E-07
b4040	5476.346	0.91841	3.15E-06	0.000503
b4067	10596.66	-0.71577	0.000166	0.015688
b4084	829.1375	0.650093	8.92E-05	0.009039

b4217	93763.7	0.652275	0.000298	0.026371
b4232	9132.087	0.686526	2.00E-05	0.002379
b4380	3997.595	1.510759	1.14E-08	2.95E-06
b4381	10456.16	1.445476	5.52E-07	0.000109
b4382	11489.95	1.082658	1.15E-05	0.001544
b4554	63775.38	2.876474	1.28E-32	2.65E-29