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Determining *Lactobacillus gasseri* Growth Curve in the Presence of Oxalate

by

Name: Janya Patterson



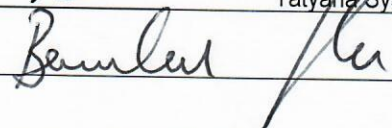
An Honors Capstone
submitted in partial fulfillment of the requirements

for the Honors Diploma to

The Honors College
of
The University of Alabama in Huntsville

Honors Capstone Director: David Cook

Program Director: Tanya Sysoeva

	Student (signature) Date 04/24/2022
 Tanya Sysoeva	Director (signature) Date 04/27/2022
	Department Chair (signature) Date 04/27/2022
	Honors College Dean (signature) Date

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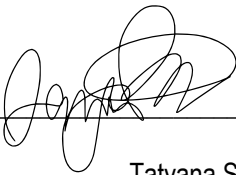

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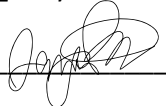
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Abstract

It is estimated that one in ten people will have a kidney stone at some time in their lives. About 11 percent of men and 6 percent of women in the United States have kidney stones at least once during their lifetime. Hyperoxaluria is characterized by the recurrence of kidney and bladder stones. Currently, only the physical removal of the stones will completely rid someone of the affliction. However, scientists are studying other preventative and therapeutic treatment methods. Recently scientists are studying how the probiotic characteristics of bacteria can positively affect the urinary microbiome. The purpose of the experiment was to determine if the presence of oxalate will affect bacterial growth, specifically *Lactobacillus gasseri*. The results indicate that bacterial growth in the presence of oxalate may be possible, proving the bacteria's oxalate degrading capabilities. The degradation of oxalate will result in an overall lower oxalate presence in the human body, reducing one's risk at hyperoxaluria. This topic needs to be further researched to provide people afflicted by Hyperoxaluria, and similar diseases, with effective and cost-efficient cures.

Introduction

Technological developments have allowed scientists to use everyday microorganisms to contribute to the growth of the medicinal and pharmaceutical fields. The properties of microorganisms: the physiology, metabolism, pathogenesis, and other genetic properties, allow scientists to use bacteria to produce antibiotics and other medically useful enzymes. More specifically, recent studies investigated the impact of Lactic Acid Bacteria (LAB) on oxalate levels. Genera of bacteria labeled as LAB are commonly *Lactobacillus*, *Leuconostoc*, *Lactococcus*, and *Streptococcus*. LAB can be found in plants and intestinal mucus of mammals. LAB are capable of converting carbohydrate substrates into organic acids, and producing a wide range of metabolites necessary for growth and reproduction (Chamberlain 2019); an anaerobic

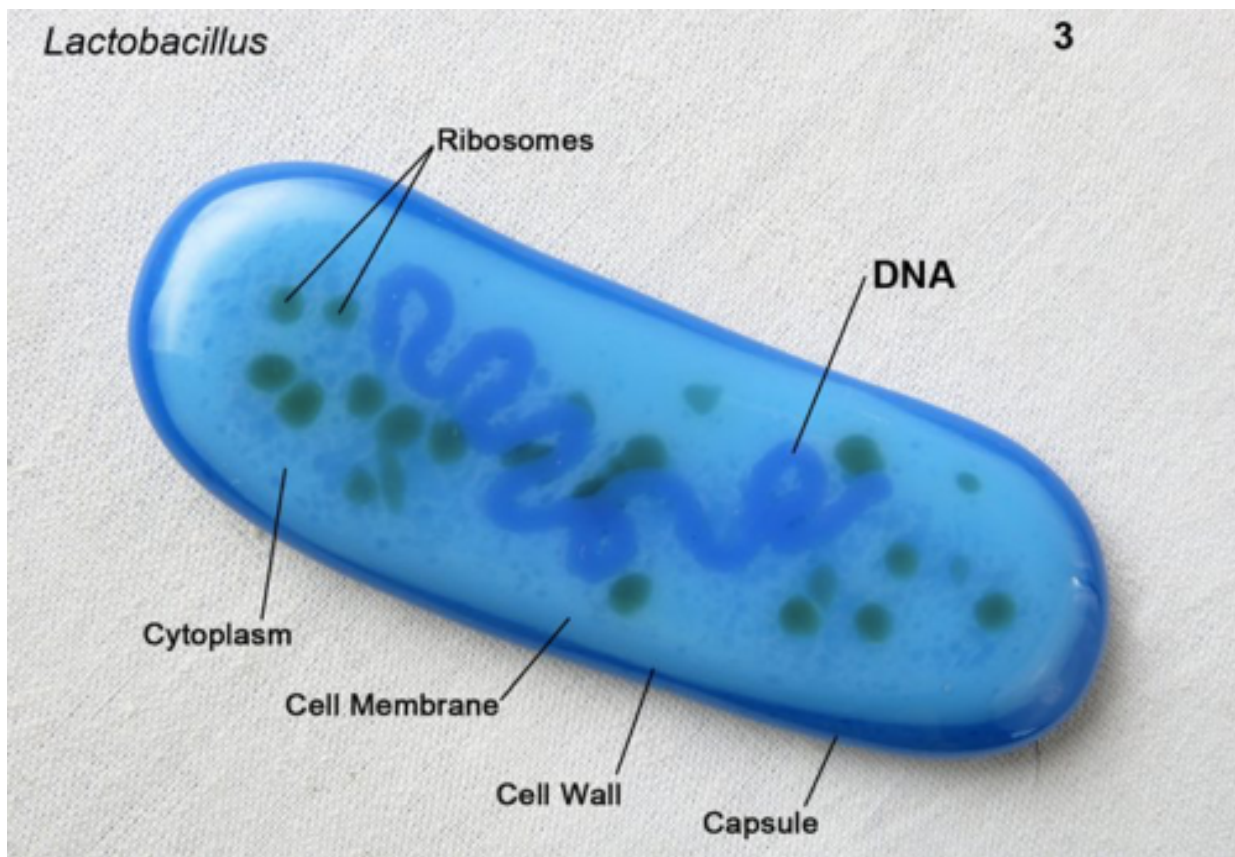


Image 1: Representation of *Lactobacillus* (Chamberlain 2019)

metabolic process. LAB has been used in methods such as healthy food preparation, and creating starter bacterial cultures: yeast, yogurt, etc. *Lactobacillus* is a rod-shaped bacterium that is mainly anaerobic, having the ability to grow or survive in the absence of oxygen. This bacterium produces hydrogen peroxide as it grows and can be commonly found in the gastrointestinal tract of animals and humans. *Lactobacilli* have been known to fight off disease causing organisms, break down food, and even absorb nutrients within different areas of the human body. Bacteria found in the gut, *Lactobacilli* and *Oxalobacter formigenes* for example, demonstrate a similar oxalate degrading mechanism as seen with oxalate decarboxylase (OxdC). *Lactobacillus plantarum* has been used as framework to constitutively produce and secrete oxalate decarboxylase while colonizing the gut to prevent the formation of kidney stones in rats. A vast amount of research has gone into studying the ability lactobacilli has to prevent CaOx stone formation. Previously, the only viable treatment therapy was the physical removal of the stones, but scientists are investigating the use of antibiotics and other medicines as viable treatment options. There is no cure for the disease, only preventative and treatment measures.

OxdC is an enzyme derived from *Bacillus subtilis*, belonging to the lyase family. A lyase breaks the bond without the means of hydrolysis or oxidation. Oxalate decarboxylase catalyzes the reaction shown in **equation 1**. It is suspected the enzyme is Manganese (Mn)-dependent that utilizes Mn (III) to degrade oxalate (Wen 2015). The exact mechanism is still unspecified.

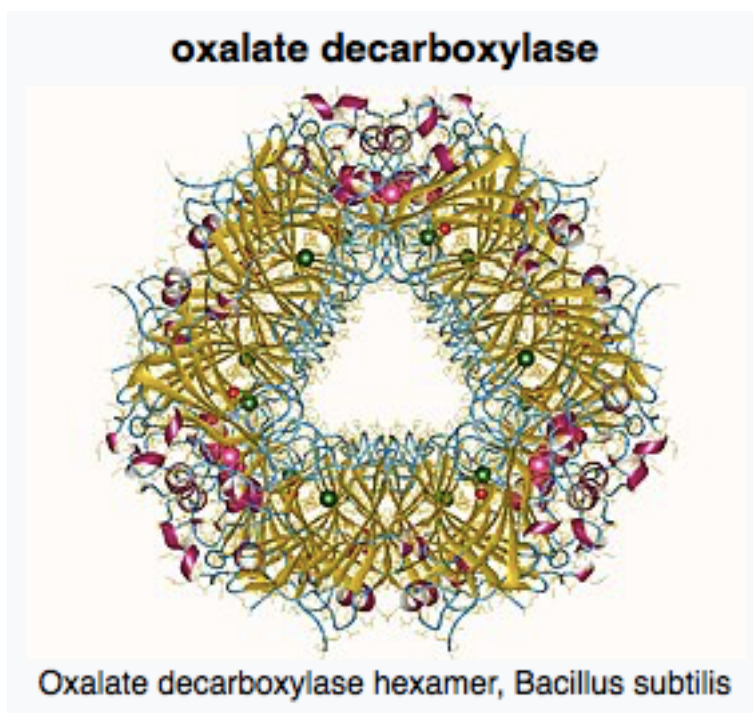
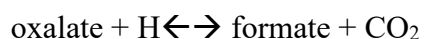


Image 2: Representation of oxalate decarboxylase (Wen 2015)



Equation 1: The reaction catalyzed by OxdC

Calcium Oxalate (CaOx) is represented with the chemical formula CaC_2O_4 . Oxalate is an unrequired nutrient found in the human body, with intense binding properties. Consumption of leafy vegetables, starches, and other types of nuts produces oxalic acid waste.

Oxalate in humans can be eliminated through excretion in urine, forming insoluble calcium oxalate and elimination in feces, or oxalate degradation by gastrointestinal (GIT) microorganisms (Abratt, 2010). When exposed to calcium, the waste can form calcium oxalate crystals in the urine. In conjunction with too much oxalate (also known as hyperoxaluria) or too little urine in the body, these circumstances result in the formation of kidney stones.

Hyperoxaluria is characterized by recurrent kidney and bladder stones that prevent waste from being properly filtered through the body, eventually leading to death. Hyperoxaluria is caused either through genetics (primary Hyperoxaluria) or by dietary habits (secondary Hyperoxaluria). Genetics is the cause of about 80% of those affected by the disease, affecting 20% of the population worldwide (Paul, 2017). Calcium oxalate crystals are the most common cause of kidney stones. Studies suggest oxalate exposure produces concentration-dependent effects on renal cell growth and viability, thus causing mitochondrial dysfunction. It has been demonstrated monocytes in patients with CaOx kidney stones have decreased mitochondrial function compared to healthy subjects. (Mejia-Pitta, 2021.)

There are many experiments with several different variables that contribute to our current knowledge of the preventative and treatment methods of CaOx stone formation. Scientists varied the types of LAB used, the growth and isolation conditions, the recombination forms of said bacteria, as well as the types of growth assay. The two most pertinent species used are *Lactobacillus acidophilus* and *Lactobacillus gasseri* (Chamberlain 2019), with oxalate degradation in the lactic acid bacteria being both species- and strain-specific. Studies have established select species of *Lactobacillus* can degrade oxalate in vitro and even decrease urinary oxalate in animal models of Primary Hyperoxaluria. However, these data results require further experimentation. Other risk factors must be considered. One in particular is if the absence of the bacteria could become a potential problem (Abratt, 2010).

The purpose of the experiment was to determine the effects oxalate has on the growth of *Lactobacillus gasseri*, and if those effects are significant enough for further experimentation. In order to test the efficacy of *lactobacilli* on oxalate degradation, a controlled cell culture was created. A control culture was necessary to compare cell growth to the cells plated in media

enriched with oxalate. Final oxalate concentration was determined using the Spectramax Id5 fluorescence machine for well plate analysis.

Materials & Methods

Sample Media Plate Preparation

The agar plate used in the experiment was a sheep blood agar plate. The nutrients found in the agar are progressive to the growth of bacteria. The blood agar was used to ensure cell growth with a reduced risk of contamination of the sample. The strain obtained, SL96 (Sysoeva Lab sample #96), was provided by Dr. Sysoeva. A sterile toothpick was used to streak the inoculation into four different sections, using a new toothpick after beginning a new quadrant. This technique is preferred to isolate single bacterial colonies throughout the agar plate. The plate was then lidded and placed in a zip lock bag with an anaerobic pack for incubation. The plate was incubated at 37° C for 24 hours.

Overnight Sample Culture Inoculation

The samples prepared for inoculation were derived from a previously streaked media plate, either a peer's or my own. The procedure was conducted in the presence of a lit Bunsen burner, to attempt to ensure a sterile working environment. A sterile working environment helps to prevent contamination of the sample. 4 mL of MRS was pipetted into two inoculation tubes. One of the tubes was sealed off and labeled as the control. A sterilized transfer loop was used to collect and distribute cells within the tube. The tubes were placed in a zip lock bag with an anaerobic pack and incubated at 37° C for 24 hours.

Oxalate Solution Preparation

100 mM of sodium oxalate (NaOxalate) stock solution was prepared first. 0.135 g of NaOxalate was dissolved in 10 mL of double distilled mQ H₂O and filter sterilized using 0.2 µm PVDF filter. A 10 mM NaOxalate solution was prepared from the 100 mM solution. 1 mL of 100 mM solution and 9 mL of sterile mQ H₂O were combined to create the stock solution.

Well Plate Preparation

Well plate analysis is used to analyze the contents of liquid cultures. In this experiment in particular, the plate reader was used to characterize the growth phases of the sample *L.gasseri* bacteria. A clear bottom, 96 well, non-treated well plate was used during the process. Bacteria growth was analyzed using the Spectramax Id5 fluorescence machine, using Softmax Pro 7.1 software. The growth conditions were consistent throughout each trial. The read type was set as kinetic absorbance with an absorbance value of 600 nm. The plate reader was set for a 24-hour timeframe, with an absorbance value measured every 15 minutes. At a constant temperature of 37°C, the well plate was shaken in an orbital shape 10 seconds before analysis began, and every 5 seconds after. **Figures 1 and 2** show the results of trials 1 and 2, respectively.

Trial #1 consisted of analyzing the growth of *lactobacilli* in conditions absent the presence of sodium oxalate. Three solutions were prepared for well plate analysis. The sample *L.gasseri* bacteria used was derived from lab mate Rachel's previously prepared MRS plate. Those samples were inoculated overnight and prepared for the well plate. The first solution was composed solely of 200 µL of MRS media. This solution was used as a reference point to confirm the viability of the media. The next solution was a 1:100 sample dilution, meaning the sample contained 180 µL of MRS culture, and 20 µL of sample culture. The final solution prepared was the 1:10 sample dilution. A separate culture solution was prepared using 900 µL of

MRS media, and 100 μL of sample culture. 20 μL of the solution, along with 180 μL of MRS media, composed the 1:10 diluted solution.

Trial #2 consisted of analyzing the growth of a single strain of *lactobacilli* in the presence of sodium oxalate. The above steps were repeated twice, the second time using carbohydrate-free MRS (cfMRS) broth, instead of MRS containing glucose. The control wells in the absence of oxalate were composed of 180 μL of MRS broth and 20 μL of mQ H₂O. The dilute wells containing oxalate were composed of 160 μL of MRS broth, 20 μL of inoculated sample cells, and 20 μL of oxalate solution.

Results & Discussion

The results are best interpreted with an idea of what is expected. The four stages of bacterial growth are the lag, log, stationary, and death phases. The lag phase indicates a temporary lack of cell growth as the cells become accustomed to the culture. The log phase is characterized by an exponential growth of bacteria. The stationary phase indicates the maximum growth has been achieved, and the bacteria are sustained. When the resources or metabolites are depleted, and cells begin to die, this signifies the death phase. A smooth “S” shaped curve demonstrated in the spectra indicates cell growth is healthy and consistent with the stages of bacterial growth. A plateau is expected in conditions not favorable to the cell group or in conditions with a lack of bacteria presence (the control groups).

Trial #1

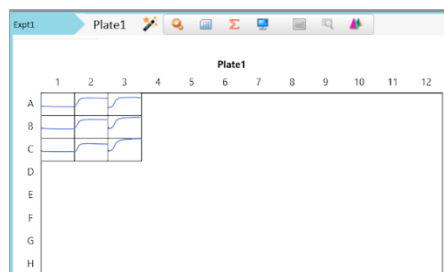


Figure 1: An image of the results produced during Trial #1, derived from the software

Wells A1-C1 are the control wells, containing only 200 μL of MRS broth. Note the expected lack of cell growth, indicating no presence of contamination. The A2-C2 wells consist of the 1:100 dilute sample. The bacteria growth is not quite optimal, seeing a lack of a log phase and an extended stationary phase. Wells A3-C3 contain the 1:10 sample dilute, exhibiting a better bacteria growth curve. **Graph 1** depicts the raw data plots representing the bacterial growth, including error bars.

Trial #2

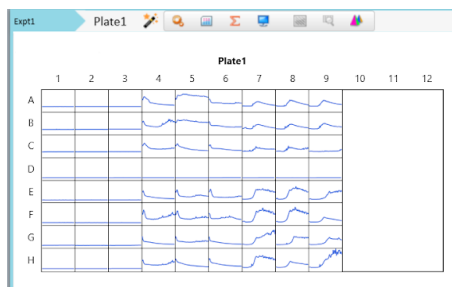


Figure 2: An image of the results produced during Trial #2, derived from the software

All the wells with lack of growth are the control wells, indicating a non-contaminated work environment. This image depicts the bacterial growth in the absence and presence of sodium oxalate, in MRS (rows A-C) and cfMRS broth (rows D-G).

Wells A1-A3 are the control wells for the MRS broth without added oxalate. Wells A4-6 and A7-9 are the 1:100 and 1:10 sample dilutions, respectively. Wells B1-B3 are the control wells for the MRS broth with added 10 mM oxalate solution. Wells B4-6 and B7-9 are the 1:100 and 1:10 sample dilutions, respectively. Wells C1-C3 are the control wells for the MRS broth with added 100 mM oxalate. Wells C4-6 are the 1:100 sample dilutions, whereas wells C7-9 are the 1:10 dilutions. Row D was skipped. Wells E1-3 are the control wells for the cfMRS broth without added oxalate, whereas wells F1-3 and G1-3 are the control wells with added 100 mM and 10 mM oxalate solution, respectively. The wells numbered 4-6 are the 1:100 dilute samples, and the wells numbered 7-9 are the 1:10 dilute samples. **Graphs 2, 3, 4, and 5** depict the raw data plots representing the bacterial growth, including error bars.

Fortunately, the control wells demonstrate the expected trendline. However, the growth curve for a few of the wells does not follow the expected “S” shaped trendline. For this reason, the results for the bacterial growth are determined to be inconclusive. This can be attributed to the amount of bacterial growth found in the initial sample inoculation. During experimentation, it was concluded that an optimal inoculation time is between 18 to 36 hours. There is a possibility the starter culture was compromised at the time of use. The media plates used were not recently made, with regards of the timing of the experiment. Also, the plates were not stored at an ideal temperature, 15 – 20° C, to preserve shelf life. The 1:10 dilutions in cfMRS broth demonstrate an extreme growth pattern, which shouldn't be observed. Bacterial growth is not supported in cfMRS broth, as observed in the 1:100 dilutions. Contamination of the sample is the key

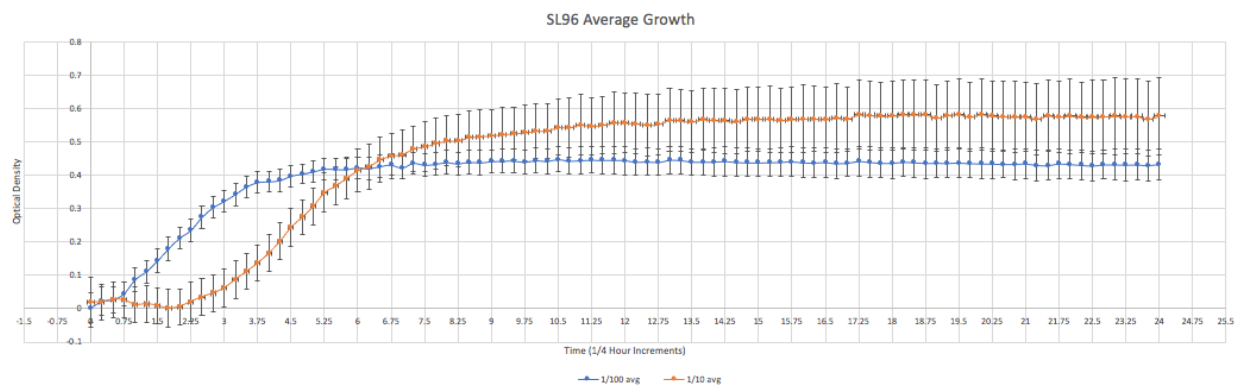
explanation for the observed trends.

Conclusion

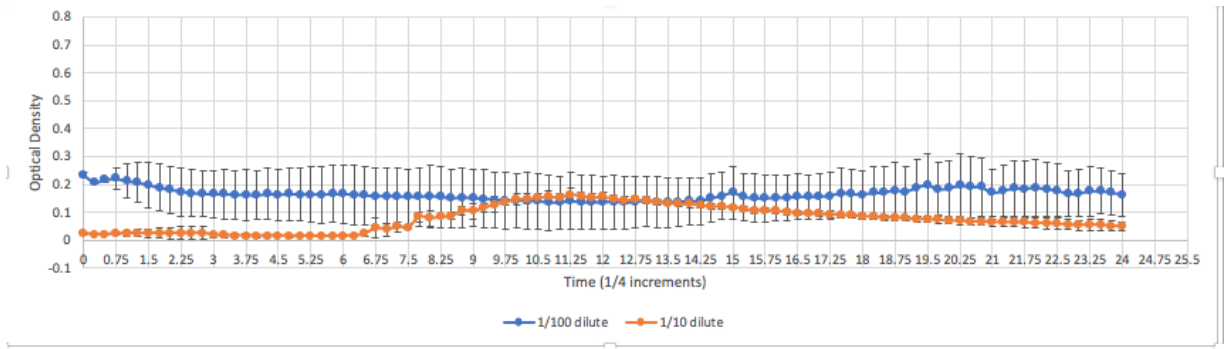
The project was intended to characterize the growth of *Lactobacillus gasseri* in the presence of oxalate and provide cause for further research. A good majority of the time was spent establishing the accuracy of the preliminary measurements, as described in Trial #1. Some genera of bacteria contribute and promote a healthy microbiome in the gut, the human vagina, mouth, etc. There are a few concurrent studies performed in the Sysoeva lab that contribute to an overall study of the probiotic properties of bacteria. In addition to my project, other colleagues of the Sysoeva lab are investigating the probiotic properties of different types of bacteria, found in different human microbiomes. For future studies, the growth data will be analyzed in conjunction with the sequencing data on urinary microbiome composition for patients on low and high oxalate diets and with whole genome analysis of lactobacilli isolates. Based on the results found, the next steps would be to test the growth properties of several other strains of lactobacillus. The final oxalate concentration would be measured. This ensures the validity and reliability of the experiment.

Appendix

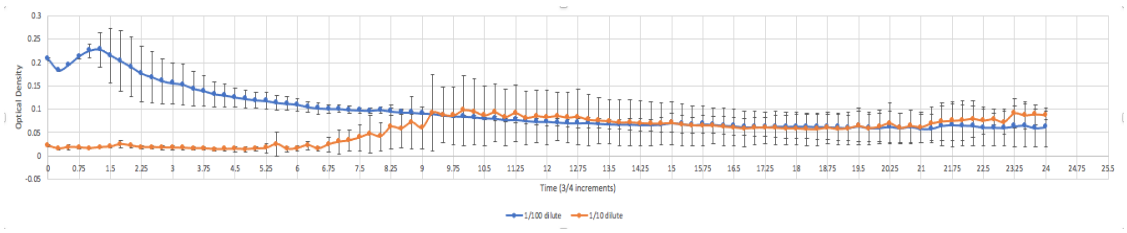
The graphs shown demonstrate the plots of the raw absorbance values of each condition's 1:100 (blue line) and 1:10 (orange line) sample dilutes. The conditions shown are the ones with bacteria grown in the presence of oxalate.



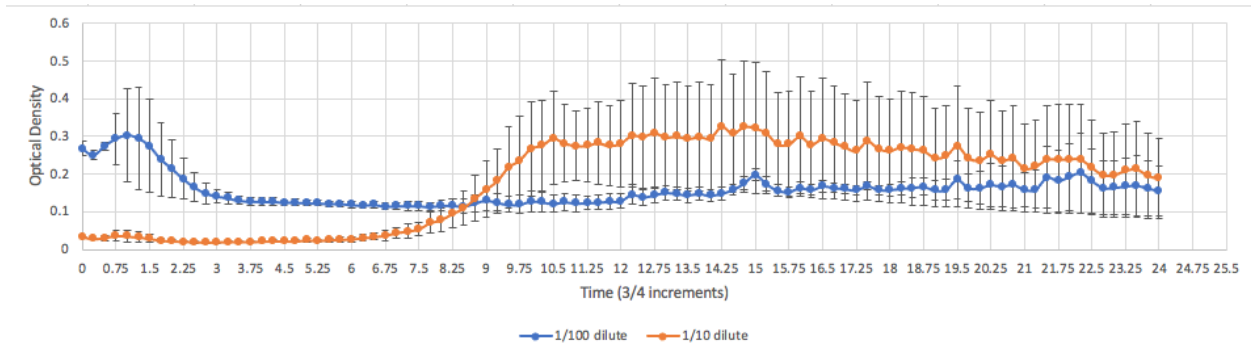
Graph 1: Comparison of preliminary bacterial growth for Trial #1



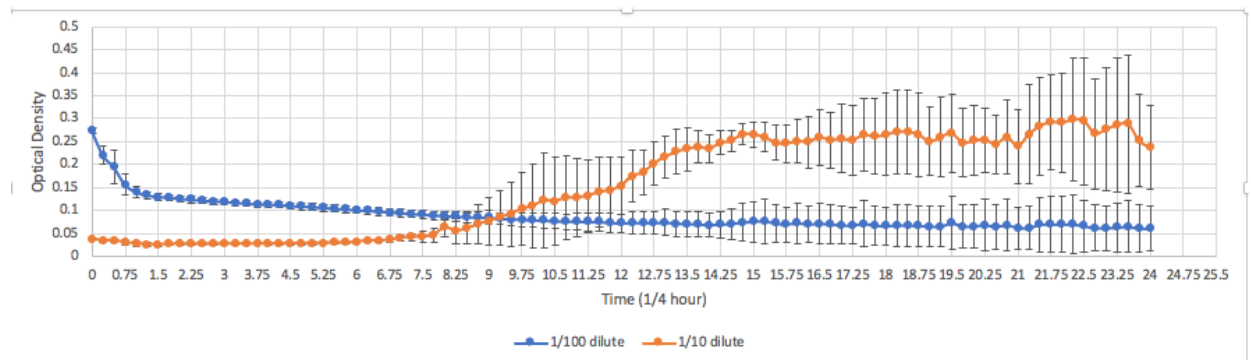
Graph 2: 1 mM oxalate concentration in MRS broth



Graph 3: 10 mM oxalate concentration in MRS broth



Graph 4: 1 mM oxalate concentration in cfMRS broth



Graph 5: 10 mM oxalate concentration in cfMRS broth

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