

# The Effects of Low-Shear Simulated Microgravity on the Soil Bacterium *Pantoea agglomerans*

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## ABSTRACT

A major stressor in the space environment is microgravity. Microgravity has profound effects on biological processes that are vital to normal functioning. This is most prevalently seen in microorganisms, which have altered growth rates and increased antibiotic susceptibility in microgravity. This is a concern for both astronauts and plants onboard spacecraft. *Pantoea agglomerans* is a soil bacterium that has been shown to be a plant growth promoter, plant pathogen, and an opportunistic pathogen to immunocompromised patients. Using the ground based microgravity analog, the Rotary Cell Culture System along with the High Aspect-Ratio Vessel, we analyzed the growth and the antibiotic susceptibility of *Pantoea agglomerans* grown in simulated microgravity. In certain parameters, we discovered an increased growth rate and no change in the antibiotic susceptibility. We found that there were differences in results when certain aspects of the protocol were altered. Further work will need to be conducted to get a better understanding of the changes in the microorganisms exposed to microgravity.

*Keywords:* HARV, Low –Shear Simulated Microgravity, microbial growth, antibiotic susceptibility

## INTRODUCTION

As the effort to establish a permanent residence in space continues, understanding the effects of a novel environment on biological organisms is vitally important. Space and the celestial bodies within, impose unique environmental stressors onto biological organisms that elicit physiological responses to adapt to the environment. Microgravity is a stressor that is felt and noticed immediately. Microgravity is described as a feeling of “weightlessness” or a “floating” sensation in space. Some of the most noticeable biological responses to microgravity occur in microorganisms. Microorganisms are great organisms to study due to their ability to quickly adapt to their environment. More importantly microorganisms are important for the health of human astronauts and their plant counterparts as they can be either beneficial or detrimental [1,2]. Previous studies have shown increased growth rates [3,4,5] and an increase in antibiotic resistance [6] in the microgravity environment. However, with limited resources and the cost of sending experiments to the International Space Station (ISS), the study of the physiological responses of microorganisms is significantly diminished. To combat this, researchers at the Lyndon B. Johnson Space Center (JSC) of the National Aeronautics and Space Administration (NASA) in Houston, Texas have developed a viable analog, the *Rotary Cell Culture System* (RCCS) (Fig. 1), licensed by Synthecon, Inc. that simulates the “sensation” of microgravity. This system simulates, or models, microgravity by rotating a High-Aspect Ratio Vessel (HARV) filled with media that contains cells

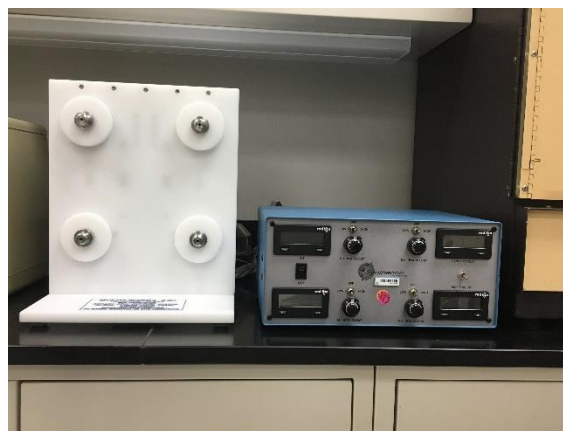


Figure 1 Rotary Cell Culture System. Synthecon Inc. Houston, TX.

rotating at a consistent speed. Preventing sedimentation, leaving the cells in a constant state of “free-fall”, and creating a low-shear environment eliciting responses to microgravity similar to those found on the ISS and other space environments. Previous studies have demonstrated the efficacy of these systems [7,8,9].

The purpose of this study is to determine the responses of *Pantoea agglomerans* (*P. agglmoerans*) to low-shear modeled microgravity (LSMMG). *P. agglomerans* is a motile gram-negative bacillus bacteria in the *Enterobacteriaceae* family of microorganisms. This microorganism has been shown to be a plant growth promoter (PGPR)[10], but in certain circumstances, it can be a plant

pathogen by competing with *Erwinia amylovora*, a bacterium that causes fire blight in plants [11,12,13], as well as an opportunistic pathogen in immunocompromised patients [14,15]. The *P. agglomerans* strain used in this study was originally isolated from the media soil on roots used for plant growth on the ISS. The objectives of this study are to determine whether microgravity has an effect on its growth as well as its antibiotic susceptibility. Because of *P. agglomerans*' opportunistic pathogenic properties, understanding how microgravity effects its growth rate and antibiotic susceptibility is important to protect the health of the plants and astronauts on the ISS, particularly because of immune system repression in astronauts [16,17]. The antibiotics chosen for this study were Ciprofloxacin, Tetracycline, and Chloramphenicol. Ciprofloxacin is typically used to treat urinary tract infections (UTI) and can be found on the ISS. One previous study showed that *P. agglomerans* had been isolated from UTIs [11,14,18]. Tetracycline and Chloramphenicol are broad spectrum antibiotics but affect the bacterial cell differently.

This study is composed of two different experiments. In order to have an accurate analog for microgravity, the HARV's must be filled completely (zero headspace) to eliminate bubbles to reduce "shear forces" that disrupt the growth of the bacteria. In addition, in order to determine the growth rate, a sample must be removed to read the optical density (OD). Removing the sample creates bubbles that need to be removed before continuance of rotation. To combat this, experiment one (Exp. 1) tests the growth rate when there is media added back into the HARV when a sample reading is taken. Experiment two (Exp. 2) tests the growth rate by rotating the HARVs to specific time points, removing them and harvesting the contents entirely. The main concern for the experiments was that the addition of media, into the HARV during the experiment, may slow the growth rate: there would be more resources for the bacteria to compete for.

## **MATERIALS AND METHODS**

**Bacterial strain.** The *P. agglomerans* strain used in this study was isolated from the media soil on roots used for plant growth on the ISS.

**Bacterial culture.** For both experiments, bacterial cells were grown in Trypticase Soy Broth (TSB), in overnight cultures, grown in shaking 125ml Erlenmeyer flasks. The overnight cultures were then adjusted to a 0.1 OD<sub>546</sub> (approx. 10<sup>8</sup> cells/ml). For experiment one, 49.5ml of sterile TSB was added to a 50ml HARV, 0.5ml of the culture was added to the HARV (approx. 10<sup>6</sup> cells per ml). Care was taken to remove bubbles and to leave zero headspace in the vessel. The HARVs were either rotated vertically at 25rpm on the RCCS (Synthecon Inc., Houston, TX) to simulate LSSMG or placed

horizontally and not rotated (static) to act as a normal gravity control. This experiment was repeated twice. The second trial featured shaking 125ml flask with the same volume of culture, for a ground control. Trial 1 ran two HARVs per condition (duplicate); trial 2 ran three HARVs per condition (triplicate) (See Appendix A). For experiment two, four time points along a growth curve of standard shaking culture, determined before experimentation, were chosen. 9.9ml of sterile TSB followed by 0.1ml 10<sup>8</sup> culture (approx. 10<sup>6</sup>) to a 10ml HARV. The HARVs were either rotated vertically at 25rpm to simulate LSSMG or placed horizontally and not rotated (static) to act as a normal gravity control. The HARVs were either rotated vertically at 25rpm to simulate LSSMG or placed horizontally and not rotated (static) (Fig. 2) to act as a normal gravity control (NG). A shaking flask culture was also used for a ground control. Each of the four time points ran in duplicate. This experiment was only ran once.

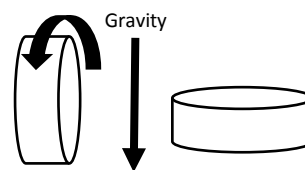


Figure 2. LSSMG - Vertical Rotation, parallel to gravity vector (Left).  
NG - Horizontal Static, perpendicular to gravity vector (Right).

**Growth curves.** For experiment one and both trials, a starting culture of 1 x 10<sup>6</sup> per ml of TSB was incubated in the HARVs at 30°C. Every hour, the HARVS were removed from the RCCS and 1ml aliquots were removed in duplicate by simultaneously adding 1ml of sterile TSB via syringes (Fig. 3) then immediately replaced onto the RCCS. In conjunction, 1ml aliquots of the shaking culture were also removed every hour. Samples were read spectrophotometrically (GENESYS™ 20, Thermo Fisher Scientific, Inc., Waltham, MA) at OD<sub>546</sub>. For experiment two, a starting culture of 9.5 x 10<sup>6</sup> per ml of TSB was incubated in the HARVs at 30°C. At time points of 1, 3, 5, 7 hours after inoculation, the HARVs were removed from the RCCS and the entire contents were harvested. 1ml aliquots were removed in duplicate and read spectrophotometrically at OD<sub>546</sub>.

**Bacterial Counts.** At each time point, bacterial culture from LSSMG, control, and shaking were serially diluted in sterile water to obtain accurate counts. 0.1ml of each diluted sample was spread on TSA plates in duplicate. Plates were incubated at 30°C for 16hrs. Countable plates were in the range of 30 to 300 colony forming units (CFU) per ml of TSB. Plate counts were only performed for experiment two. However, for starting concentration confirmation, plates counts were performed for both experiments.

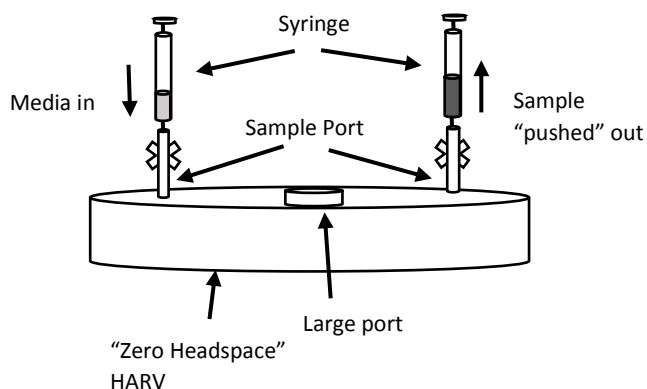


Figure 3 Diagram of Exp. 1 HARV. The addition of media when taking sample readings.

**Antibiotic Susceptibility.** For antibiotic susceptibility testing, the Kirby-Bauer Antibiotic Susceptibility test was utilized. The chosen antibiotic disks were Ciprofloxacin (5µg), Tetracycline (30µg), and Chloramphenicol (30µg) (BD, Franklin Lakes, NJ). Time point three was chosen for this test due to its average OD reading of 0.208, 0.199, and 0.161 for the LSSMG, control and shaking, respectively, being near the 0.5 McFarland standard. A sterile cotton swab was submerged into the sample and spread across Mueller-Hinton Agar plates (Carolina Biological Supply Company, Burlington, NC) to create lawns of bacteria in duplicate. The antibiotic disks were placed in a row in duplicate. The plates were incubated at 37°C for 16hrs. The zone of inhibition (ZOI) was measured in millimeters (mm) and compared to the standards ZOI for each antibiotic from the Clinical Laboratory Standards Institute (CLSI) MS100 for the *Enterobacteriaceae* family (Table 1). The procedure was initially run on *E. coli* ATC25922 for quality control. This test was only run once on experiment two.

Table 1 CLSI M100 zone of inhibition standards for Tetracycline, Ciprofloxacin, and Chloramphenicol for the *Enterobacteriaceae* family of bacteria.

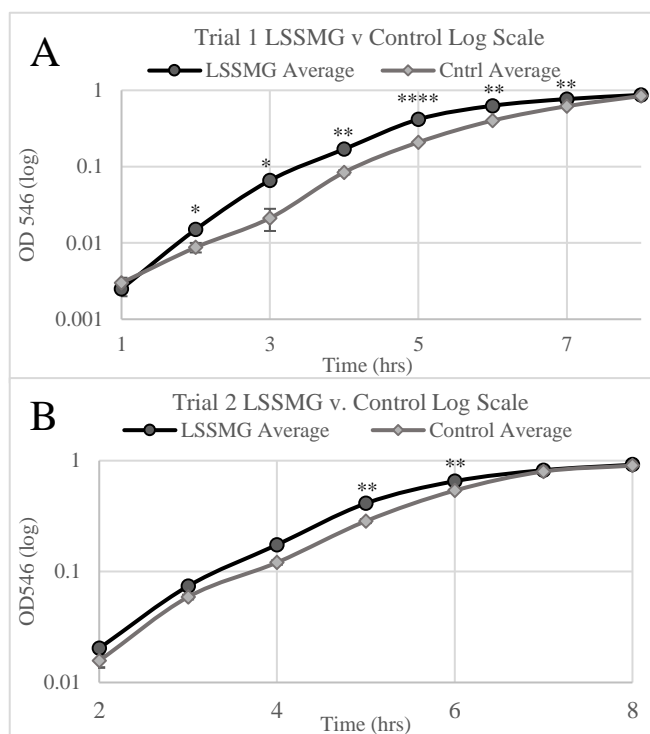
Antibiotic	Susceptible	Intermediate	Resistant
Tetracycline (30µg)	≥15mm	12-14mm	≤11mm
Ciprofloxacin (5µg)	≥21mm	16-20mm	≤15mm
Chloramphenicol (30µg)	≥18mm	13-17mm	≤12mm

**Statistical Analysis.** To determine statistical significance between the conditions of the experiments, an unpaired student T-test and a one-way ANOVA analyses were conducted on the results (GraphPad Prism, )

## RESULTS AND DISCUSSION

**Growth Curves in LSSMG and NG compared.** Two trials were completed for experiment one. In trial one, we only compared LSSMG and NG HARVs in duplicate without a shaking culture (Fig. 4A). Trial two compared LSSMG and NG, along with a shaking culture, in triplicate (Fig. 4B). Initially, trial one yielded statistically significant OD readings throughout the curve, implying a significant increase in the growth rate. However, the trial was run in duplicate, indicating a somewhat unreliable trial. In contrast, in trial two, at hours 5 and 6, there was a statically significant increase in the growth rate in LSSMG when compared to the control.

Figure 4 Experiment 1; Trial 1 and 2 growth curves. Each point along the growth curve were statistically analyzed by a student T test and one-way ANOVA. \*=*p* < 0.05, \*\*=*p* < 0.01, \*\*\*=*p* < 0.001, \*\*\*\*=*p* < 0.0001



This trial was run in triplicate, indicating reliability in the experiment. When superimposing the trials together (See Appendix B), the curves were found to be very similar to each other. The similarity of the curves indicates repeatability in the trials. In experiment one, there was a statistically significant increase in the growth rate in the LSSMG condition, most notably in trial two. However, in order to truly determine if there was a difference between the two conditions, more trials must be completed.

For experiment two, only one trial was completed. In this trial, the growth curves of all three conditions; LSSMG, NG and a shaking ground control flaks, were all very similar

with no statistical differences (Fig 5). At time point 5, there was a slight increase in the growth rate. However there was significant error at that point for that condition, due to a bubble that had formed during rotation. These results are inconclusive due to one trial and run in duplicate.

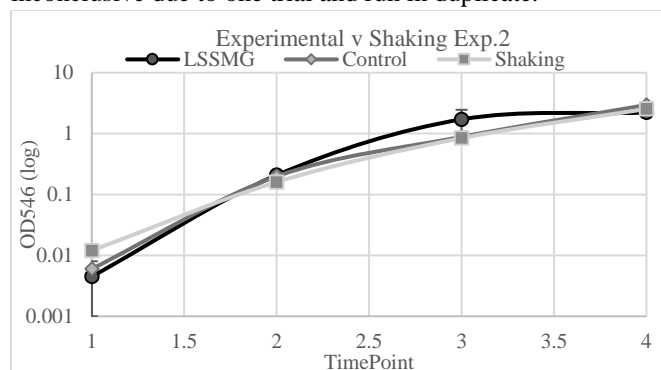


Figure 5 Experiment 2; Trial 1 growth curve.

Because of the nature of this experiment, the limited amount of HARVs hindered the ability to repeat and generate more trials. In order to determine an actual growth curve, without adding media to the vessel, this experiment will have to be repeated.

**Bacterial plate counts.** It was found that there were no significant changes between the plate counts for each time point in experiment two (See Appendix C). Unfortunately, bacterial counts were only conducted on experiment two and only completed one time. It was also noticed that towards the later time points, the samples were not diluted far enough to get an accurate plate count to determine changes of growth in each condition.

**Antibiotic Susceptibility.** After experimentation, it was found that there were very little differences in the ZOI of for each condition (Table 2). When comparing the data to the standards, all three conditions were well over the susceptible indicator. Like the bacterial counts, this test was conducted in experiment two and was only run once without a repeated trial. Also, the bacteria and antibiotics were plated at the second time point (3hrs into experimentation), indicating that the bacteria may have not had time to develop or adapt to the environment.

Table 2 ZOI of each condition for each antibiotic, with a qualitative assessment letter. S=susceptible, I=intermediate, R=resistant.

Condition	Tetracycline	Ciprofloxacin	Chloramphenicol
LSSMG	21.1mm(S)	34.4mm(S)	24.4mm(S)
Control	22.4mm(S)	34.9mm(S)	24.3mm(S)
Shaking	22.4mm(S)	35.4mm(S)	24.9mm(S)

**Tale of two experiments.** After experimentation, we found that there were some differences between the two ways the experiment was conducted. For example, in the growth

curves, we noticed that there was a statistically significant increase in the growth rate between LSSMG and control were prevalent in the experiment where sterile media was added to the vessel when an OD reading was taken and then placed back onto the RCCS system. In contrast, there were no significant differences between LSSMG and the control conditions in experiment two where sterile media was not added back into the vessel and was completely harvested. Multiple factors could have contributed to this. For instance, the volume of the vessel may have contributed, experiment one used 50ml HARVs and experiment two used 10ml HARVs.

### FUTURE WORK

To better understand the true effects of a Low-Shear simulated microgravity environment on *P. agglomerans*, these experiments will need to be repeated due to the minimal amount of trials and the usage if duplicate vs triplicate. Also, it should also be suggested that bacterial cultures grown in HARVs be grown overnight in experimental conditions i.e. in the HARV and rotating or control, to get true adaptation to the environment. More work will also have to conduct on the two experiments, the addition of media when readings are taken or harvesting the entire vessel. Lastly, the antibiotic susceptibility analysis should also be conducted on experiments where media is added in to the HARV.

### ACKNOWLEDGEMENTS

The authors would like to thank Anna Maria Ruby and Dr. Ye Zhang of NASA Kennedy Space Center, for providing the opportunity to conduct this project, invitation to the center, the RCCS system, and technical advice. We also thank Mary Hummerick and Dr. Christina Khodadad of the AECOM LASSO contract at KSC for their generous support and providing lab space, resources, computer programs, and the strain of *P. agglomerans*. Additionally, we also thank Jeff Richards of the AECOM LASSO contract at KSC for supplying resources such as the HARVs and materials for the antibiotic susceptibility. Lastly, this project was supported and funded by the NASA North Dakota Space Grant Consortium.

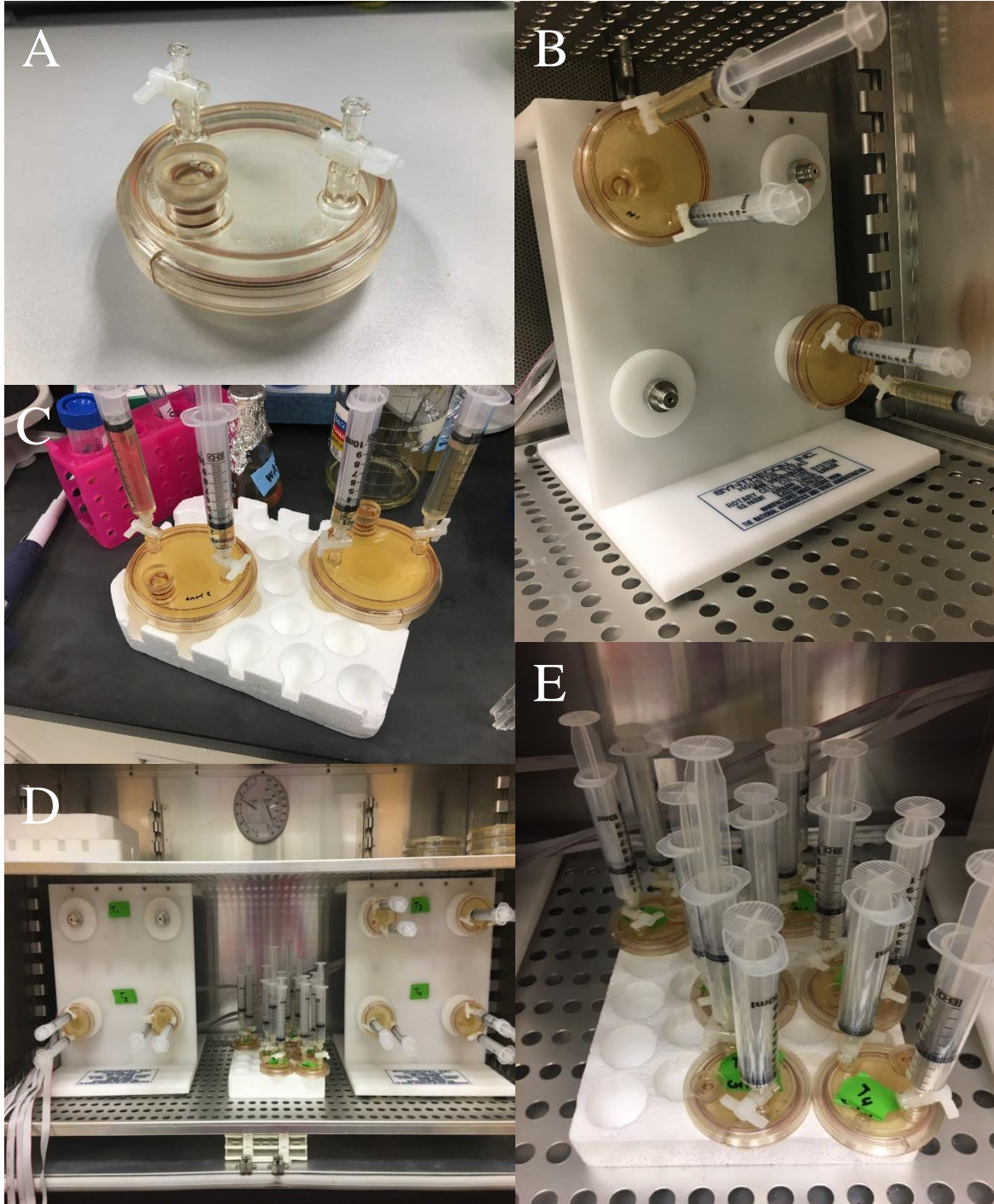
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**Appendix A**

Figure 6 The High Aspect-Ratio Vessel (HARV)(A). Experiment one, trial one, addition of media when sample is removed for optical density reading (LSSMG – B, Control - C). Experiment 2, time points of 1h, 3h, 5h, 7h. No addition of media for sample reading, harvesting entire vessel (LSSMG – D, Control – E).

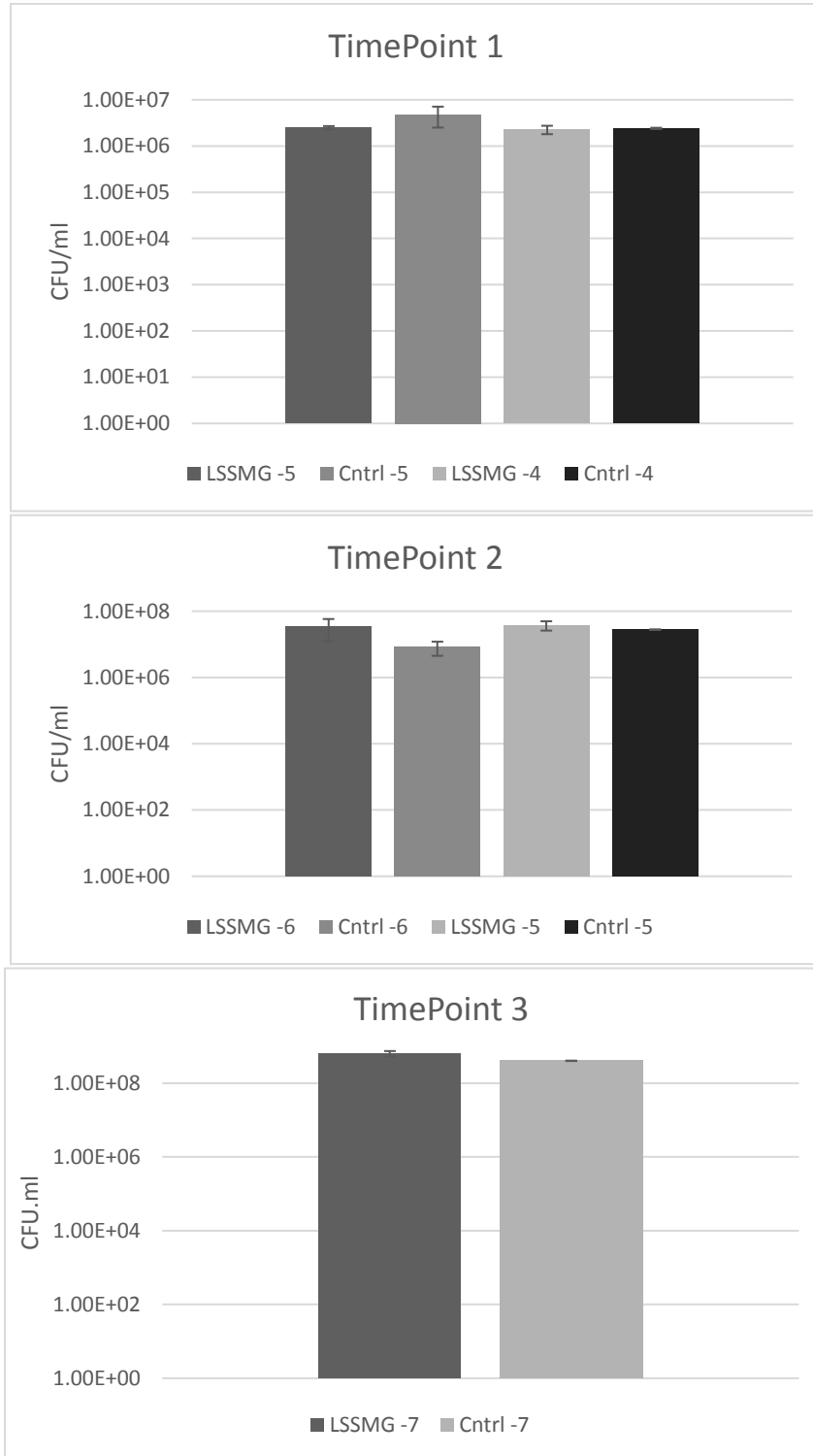


**Appendix B**



Figure 7 Experiment 1, Trials Compared. Indicating Repeatability.

**Appendix C**



*Bacterial Plate Counts. Counted for each time point in duplicate. Graphs represent average of duplicates in each condition. The number next to the condition represents the dilution.*