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Article

Intra and interspecies control of bacterial growth through extracellular extracts

Bailey Howe[1], Michael Estrella[1]
[1] Brooklyn Technical High School, Brooklyn, New York

SUMMARY

A variety of bacterial species colonize the human body, many of which reside in the gut microbiome. The disruption of the gut microbiome is linked to several digestive issues and chronic health conditions, such as obesity, *Clostridium difficile* (*C. difficile*) infections and colorectal cancer. Bacteria utilize extracellular quorum sensing molecules to communicate, which impacts bacterial growth, resistance to antibiotics, and differential expression of genes. Influence and control over these systems is of clinical importance in treating bacterial infections, such as by modifying a patient's gut microbiome. This study measured the impact of bacteria-free supernatants collected from *Escherichia coli* (*E. coli*), samples from the bacterial environments likely containing quorum sensors, in the death and log phases on the growth of new cultures of *E. coli* and *Enterobacter aerogenes* (*E. aerogenes*). Exposure to *E. coli* death phase clarified supernatant inhibited *E. coli* growth by 21.41% - 81.24% and *E. aerogenes* growth by 19.43% - 58.62%. While *E. coli* log phase clarified supernatant induced *E. coli* growth by 15.08% - 52.61% and *E. aerogenes* growth by 29.93% - 107.52%. A combination of these supernatants could potentially be used to fine-tune population sizes of bacteria in the gut microbiome, rebalancing an altered gut microbiome. Future experiments could characterize the macromolecules within the clarified supernatants, explore their degradation pathways, and how they interact with each other.

INTRODUCTION

The human microbiome contains trillions of diverse bacteria, many of which reside in the gut (1, 2). Disruption of the gut microbiome is implicated in many serious health complications, such as *Clostridium difficile* (*C. difficile*) infections, inflammatory bowel disease, irritable bowel syndrome, colorectal cancer, and obesity (1, 3-7). The importance of maintaining the gut microbiome in human health has created a need for research on methods to sustain healthy levels of bacterial populations.

Bacteria experience four main stages of growth: the lag, log, stationary, and death phases (8). During the log and death phase, the bacteria population changes far more than in the other two stages, with bacteria experiencing rapid growth in the log phase and widespread death in the death phase (8). Quorum sensing is a common ability of bacteria to sense environmental conditions, including the presence of other organisms, and alters their gene expression accordingly (9). These sensors allow unicellular bacteria to exhibit multicellular behavior by interacting with each other and their environment. Quorum sensors are implicated in the increased fitness of pathogenic bacteria through the formation of biofilms, resistance to antibiotics, and expression of viral genes (9-12). Other quorum sensors, such as the extracellular death factor, which is a linear pentapeptide produced in bacteria, have been found to severely inhibit bacterial growth and lead to the death of bacterial populations (13). Selective inhibition and induction of quorum sensors could prove to be a powerful tool against disease and in the maintenance of a healthy microbiome.

We hypothesized that adding media from *Escherichia coli* (*E. coli*) in the death phase would significantly inhibit the growth of a new culture of *E. coli*. We reasoned that the bacteria present in the new culture would process and interpret these secreted factors as an indicator of a hostile or nutrient-deficient environment, and therefore decrease their growth rate to fit this environment. We hypothesized that this change would be temporary, lasting for only a few hours, because the bacteria would break down these inhibitory molecules and maximize their growth according to the actual growth conditions and not the false ones signaled by the added secreted factors. Conversely, we predicted that adding clarified media from *E. coli* in the log phase to a new culture of *E. coli* would increase growth due to a perceived safe and nutrient-rich environment, but much like with the media from the death phase, this would also only cause a temporary shift as the bacteria adjust their growth rate to best suit their environment. Lastly, we hypothesized that these different types of clarified media would have similar effects on cultures of another bacterial species; however, the effects of the extracellular extracts would likely be lesser due to their foreign nature. We chose *Enterobacter aerogenes* (*E. aerogenes*) to measure the interspecies effects of this media due to its shared status as a member of Enterobacteriaceae and ability to grow well on the same media as *E. coli*. We found that the secreted factors collected from *E. coli* did influence its growth as predicted at some points during 23.8 hours of growth. Similarly, the secreted factors of *E. coli* did influence *E. aerogenes* growth at some time intervals. However, *E. aerogenes'* response to *E. coli's* secreted factors was not always lesser than *E. coli*'s response to its own secreted factors.

RESULTS

We first grew *E. coli* for 6 hours in at 37ºC in our aerobic laboratory conditions to establish a growth curve. From this curve, we determined the log phase of growth for subsequent experiments. We were able to fit *E. coli* growth to a sigmoid curve with an R2 value of 0.9853. We found *E. coli* experienced the log phase growth from 2-6 hours of growth when incubated. (**Figure 1**). This information dictated

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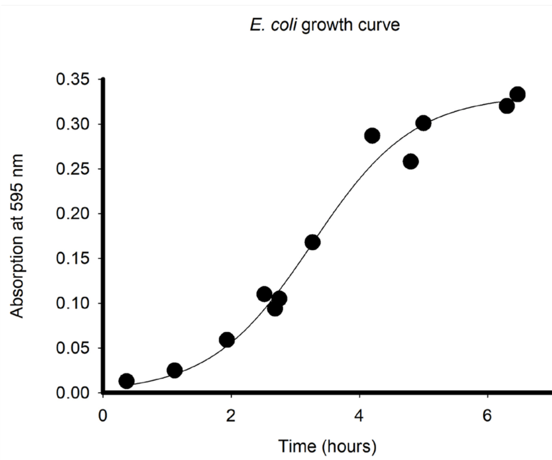

Figure 1. Growth of E. coli (n=1) in our laboratory conditions. A sigmoid curve was fit to the data with an R2 value of 0.9853. The log phase was observed to occur from 2-6 hours of growth. These time values were then used for all subsequent experiments using log phase supernatants.

how long we grew *E. coli* to collect our log phase extracellular extract. Additionally, *E. coli* appeared to grow more slowly in our laboratory than others (8).

We found that the addition of clarified media collected from *E. coli* experiencing the log phase of growth significantly increased *E. coli* growth at most time intervals (2.5 and 23.8 hours), with experimental solutions of *E. coli* experiencing 115.08% of normal growth at 2.5 hours, 122.77% at 4.8 hours, and 152.61% at 23.8 hours, with standard errors of the mean of 9.37%, 6.77%, and 29.80%, respectively (**Figure 2**). We found that this media had a significant effect on *E. aerogenes* growth as well at 2.5 hours, with experimental solutions experiencing 207.52% of normal growth at 2.5 hours, 129.93% at 4.8 hours, and 190.81% at 23.8 hours, with standard errors of the mean of 24.19%, 9.42%, and 2.97%, respectively. (**Figure 3**).

We found *E. coli* growth to be significantly inhibited by clarified media collected from *E. coli* in the death phase at 2.5 and 4.8 hours, with experimental cultures growing only 18.76% of normal growth at 2.5 hours, 39.09% at 5 hours, and 78.59% at 23.8 hours, with standard errors of the mean of 3.28%, 7.24%, and 18.05%, respectively **(Figure 2).** Similarly, we found *E. aerogenes* growth was significantly inhibited by the same *E. coli* death phase clarified supernatant at the same time intervals, with experimental cultures experiencing 41.38% of normal growth at 2.5 hours, 50.63% at 5 hours, 80.57% at 23.8 hours, with standard errors of the mean of 4.98%, 4.57%, and 3.81%, respectively. (**Figure 3**).

E. aerogenes was far more stimulated at 2.5 hours by *E. coli* log phase clarified supernatant (LS) and less inhibited by *E. coli* death phase clarified supernatant (DS) at 2.5 hours than *E. coli*. Additionally, there were no significant differences between the two species when exposed to either extract at 4.8 and 23.8 hours, suggesting equally strong inter- and intraspecies growth effects of DS and LS at those time values **(Figure 4)**. We summarized our results on the stimulatory effect of LS and the inhibitory effect of DS and their potential applications in a model **(Figure 5)**.

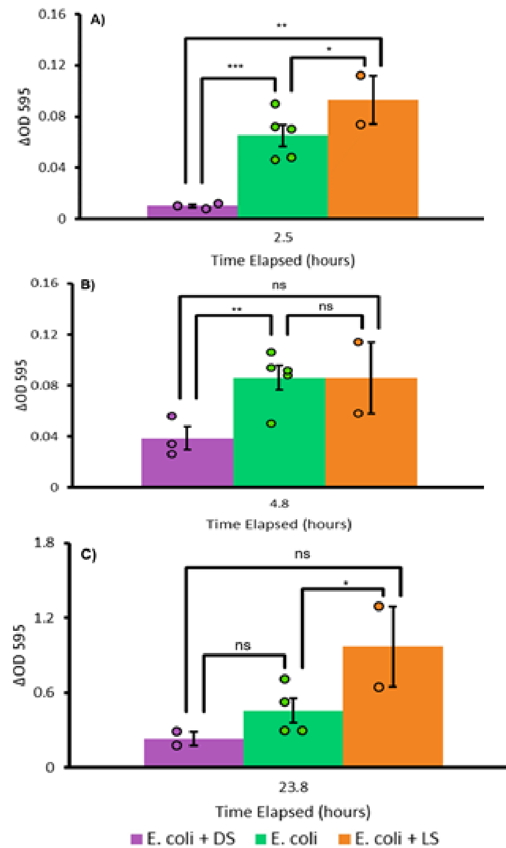

Figure 2. Impact of E. coli extracellular extracts on E. coli growth. Comparison of E. coli extracellular extracts on the growth of new cultures of E. coli growth at a. 2.5 hours b. 4.8 hours, c. 23.8 hours. The calculated P value (two tailed, unpaired T- test) for E. coli + DS (n=3 at times t=2.5 and 4.8 hours, n=2 at 23.8 hours) vs untreated E. coli (n=5) is 0.00119 at 2.5 hours, 0.00797 at 4.8 hours, and 0.110 at 23.8 hours. The calculated P value for E. coli + LS (n=2) vs untreated E. coli is 0.0817 at 2.5 hours, 1 at 4.8 hours, and 0.0540 at 23.8 hours. The calculated P value (two tailed, unpaired T- test) for E. coli + DS vs E. coli + LS is 0.00503 at 2.5 hours, 0.0708 at 4.8 hours, and 0.0770 at 23.8 hours. A single asterisk represents a p-value below 0.05, two asterisks represents a p-value below 0.01, and 3 asterisks represents a p-value below 0.001. Error bars represent one standard error of the mean.

During our preliminary experiments, it quickly became apparent that the freshness of our extracellular extracts was a determining factor in how they affected growth. We decided to test storing DS at 37°C, 25°C, 4°C, and -20°C before adding it to new cultures of *E. coli* to measure the impact of storage at different temperatures. *E. coli* was stimulated at 2.8 hours by DS stored for 10 days at all four temperature conditions, with experimental groups experiencing 135.29% - 473.53% of growth. At 4.8 hours there was similar stimulation of all groups except *E. coli* + 25°C DS, and these groups experienced 106.06% - 254.55% of normal growth. *E. coli* + 25°C DS experienced 89.39% of normal growth at this time value. At 23.8 hours, all groups experienced inhibition with only 67.81% - 80.14% of normal growth. (**Figure 6**).

DISCUSSION

In our study, we first established a growth curve for *E. coli* in our laboratory conditions and used it to identify the precise

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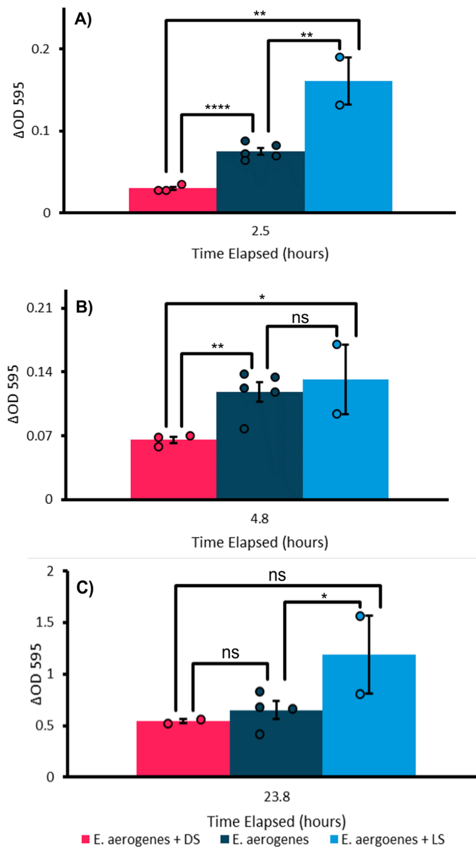

Figure 3. Impact of E. coli extracellular extracts on E. aerogenes growth. Comparison of E. coli extracellular extracts on the growth of new cultures of E. aerogenes growth at a. 2.5 hours, b. 4.8 hours, c. 23.8 hours. The calculated P value (two tailed, unpaired T- test) for E. aerogenes + DS (n=3 at times t=2.5 and 4.8 hours, n=2 at 23.8 hours) vs untreated E. aerogenes (n=5) is 0.000136 at 2.5 hours, 0.00541 at 4.8 hours, and 0.227 at 23.8 hours. The calculated P value (two tailed, unpaired T- test) for E. aerogenes + LS (n=2) vs untreated E. aerogenes is 0.00585 at 2.5 hours, 0.312 at 4.8 hours, and 0.0558 at 23.8 hours. The calculated P value for E. aerogenes + DS vs E. aerogenes + LS is 0.00460 at 2.5 hours, 0.0515 at 4.8 hours, and 0.115 at 23.8 hours. A single asterisk represents a p-value below 0.05, two asterisks represents a p-value below 0.01, three asterisks represents a p-value below 0.001, and four asterisks represents a p-value below 0.0001. Error bars represent one standard error of the mean.

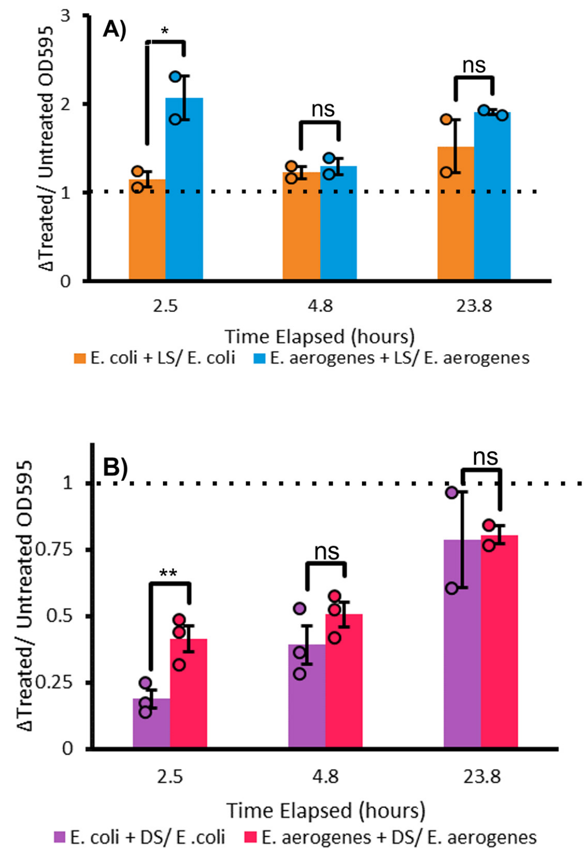

Figure 4. Inter- vs intraspecies effects of E. coli extracellular extracts. a. Comparison of E. coli log phase supernatant (LS) impact on the growth of new cultures of E. coli and E. aerogenes (n=2). The dotted line represents a 1:1 ratio of growth indicative of the extracellular extracts not impacting bacterial growth. The calculated P values (two tailed, unpaired T- test) for E. coli + LS / untreated E. coli vs E. aerogenes + LS / untreated E. aerogenes, at 2.5 hours is 0.0356, at 4.8 hours is 0.301, and at 23.8 hours is 0.162. b. As above, but with E. coli clarified death phase supernatant (DS) in place of LS. The dotted line represents a 1:1 ratio of growth indicative of the extracellular extracts not impacting bacterial growth. The calculated P value (two tailed, unpaired T- test) at 2.5 hours is 0.00938, at 4.8 hours is 0.126, and at 23.8 hours is 0.471. For time t=2.5 hours and t=4.8 hours, n=3, and for time t=23.8 hours, n=2. A single asterisk represents a p-value below 0.05 and two asterisks represents a p-value below 0.01. Error bars represent one standard error of the mean.

time of the log phase during growth to purify log quorum sensors from, which was identified as 2-6 hours of growth in our laboratory conditions. We then investigated the impact of media collected from *E. coli* in the log and death phases on the growth of new cultures of *E. coli* and *E. aerogenes* to measure intra- and interspecies growth of communication molecules present during these growth phases, respectively. These solutions were found to have a significant impact on the growth of new cultures of both *E. coli* and *E. aerogenes* with LS significantly increasing the growth rate of both species at 2.5 and 23.8 hours and DS significantly inhibiting the growth rate of both species at 2.5 and 4.8 hours.

Both species of bacteria in our study exhibited increased growth when exposed to *E. coli* log phase clarified media. Contrary to our hypothesis, *E. aerogenes* displayed a greater growth rate when exposed to this solution at all measured time intervals than *E. coli*. Additionally, *E. aerogenes* exposed to *E. coli* log phase clarified media displayed an extreme amount of growth 2.5 hours into the experiments. There was no significant difference in the growth of *E. coli* and *E. aerogenes at* 4.8 and 23.8 hours into the experiment, suggesting equally powerful inter- and intraspecies effects on bacterial growth of these extracellular extracts. Future experimentation could examine the mechanisms and reasoning behind this vast difference in growth at 2.5 hours, which could perhaps be an adaptation of *E. aerogenes* to compete with nearby *E. coli*. The data we collected partially supported the primary research hypothesis, as growth of both species of bacteria was increased due to exposure to the *E. coli* log phase extracellular extract. However, this change was not temporary

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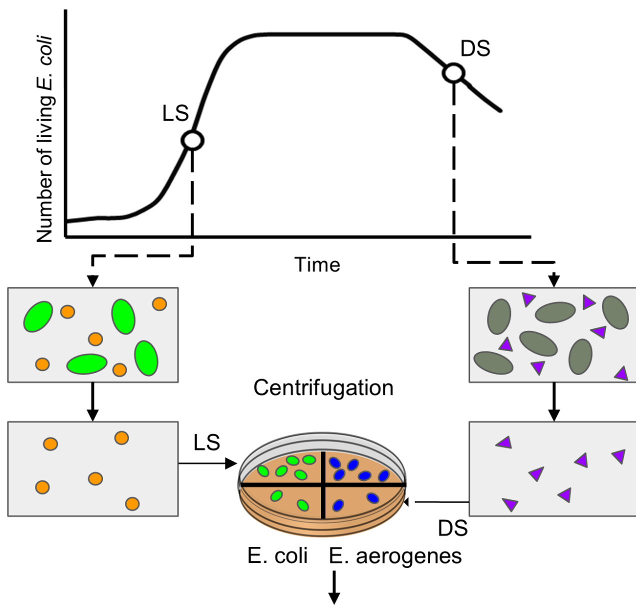

- Can improve gut microbiome diversity and fine tune bacterial populations to improve health
- Future experiments can explore specific quorum sensors interactions between critical bacterial populations and methodology for maximum health benefits

Figure 5. Model of E. coli extracellular extract inter- and intraspecies growth effects. Diagram displaying the stimulatory effects of extracellular extracts collected from E. coli experiencing the log phase of growth on both E. coli and E. aerogenes and the inhibitory effect of extracellular extracts collected from E. coli on the aforementioned species of bacteria. The implications of both extracts in improving human health are also discussed briefly, as are future experiments that can be conducted. Light green ovals represent E. coli, while dark green ovals represent E. aerogenes. Orange circles stand in for LS while purple triangles model DS.

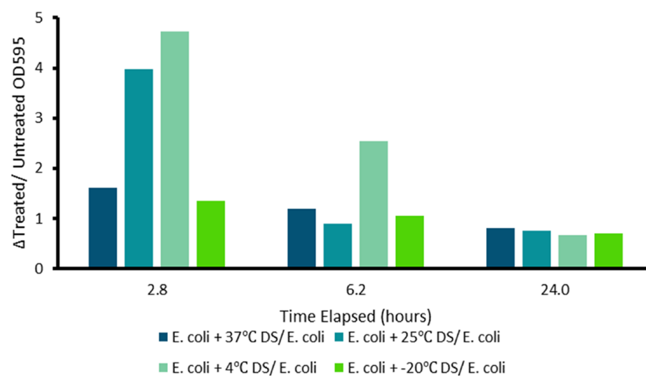

Figure 6. Impacts of storage in different temperature conditions on E. coli clarified death supernatant. Comparison of growth of new cultures of E. coli after exposure to E. coli death phase clarified supernatants stored for 10 days at 37°C, 25°C, 4°C, -20°C (n=1).

and was observed at all measured time intervals. Surprisingly, *E. aerogenes* also experienced more growth at all measured time intervals than *E. coli* when exposed to the same quantity of LS. The data suggests that differential growth rates of bacterial populations can be achieved through the molecules within these extracts, which could be useful in manipulating the gut microbiome to promote its health. It is important to note that the small sample size (n=2) may limit the predictive nature of these findings due to limited statistical significance and more experimentation is needed to confirm the effects of this extract we observed.

Death phase clarified media collected from *E. coli* limited the growth of both *E. coli* and *E. aerogenes*, although the effect was greater on *E. coli* than on *E. aerogenes* at all time intervals. Our data supports the primary research hypothesis that bacteria growth was slowed by the addition of these extracellular extracts. Additionally, *E. coli* was significantly more inhibited by its own death phase extracellular extract 2.5 hours into the experiment than *E. aerogenes*, however the inter- and intraspecies effects of these extracts were non-significantly different 4.8 and 23.8 hours into the experiment (**Figures 2-3**). Unexpectedly, the inhibitory effect of these molecules was not overcome after 23.8 hours in either species of bacteria. This finding is contradictory to our original hypothesis and indicates that the effects of extracellular extracts collected from *E. coli* experiencing the death phase persist for long periods of time. This may have implications for the treatment of gut microbiome imbalance and diseases, as it is valuable to know how long DS will affect growth if it is used therapeutically. This will determine how long such a treatment will be effective for and will influence time between doses. At time t=23.8 hours, a small sample size (n=2) may possibly limit the validity of our data at that time value and the predictive nature of our conclusions. Additionally, it is important to note that it is possible that the extracellular extract collected from *E. coli* experiencing the death phase of growth still contained some nutrients, which would increase the growth of bacteria grown in this extract. Future experiments could aim to eliminate this variable by removing these nutrients.

Clarified supernatant collected from *E. coli* in the death phase was found to increase bacterial growth in DS stored at all 4 temperature conditions (37°C, 25°C, 4°C, and -20°C) or 10 days. The difference in the effects of fresh DS and DS stored at these four temperature conditions indicates that any of the studied storage methods of extracellular extracts are ineffective at preserving them properly and that these extracellular extracts are likely sensitive to storage temperature. It is also possible that there are inhibitory and stimulatory molecules present in the death phase clarified media that interact with each other and can aid in the survival of a population in a stressful environment. The inhibitory molecules may kill off a large portion of the population before being inhibited themselves by stimulatory molecules that promote the growth of the surviving subset of the original population of bacteria. Future experimentation is necessary to fully understand and outline the effects of extracellular extracts from different stages of growth, what molecules they contain, how these molecules are degraded, and possible interactions with molecules from other extracellular extracts. Our study presents a working model for these interactions to be tested, in which the *E. coli* log phase extracts and death

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phase extracts stimulate and inhibit growth respectively **(Figure 5)**. The extracts, and others like them, could stimulate the growth of species that are present in too low quantities and inhibit those present in too high quantities. It is possible these extracts could be utilized to rebalance a disrupted gut microbiome to treat the numerous associated, often chronic, conditions.

MATERIALS AND METHODS

Bacterial Strains

The strains of bacteria utilized in this study were *Escherichia coli* K-12 (Carolina Biological Supply, Item #155068) and *Enterobacter aerogenes* (Carolina Biological Supply, Item# 155031). These strains are BSL-1 and their safe handling was accomplished through the guidelines posted on the atcc.org website.

Growth Curve

To establish a growth curve for *E. coli*, samples of *E. coli* were grown aerobically in a liquid culture in a static incubator at 37°C for 6.5 hours. The sample's optical density at 595 nanometers was measured approximately every 40 minutes using a spectrophotometer. Data were analyzed using SigmaPlot to create a trendline and time values were determined for expected phases of bacterial growth.

Log and Death Extracellular Extracts

Samples of *E. coli* were grown aerobically at 37°C in an incubator for either 3 days (72 hours) to reach the death phase or for 5 hours to reach the log phase. The cultures were then collected and centrifuged at 4500 RPM for 5 minutes to separate bacteria from surrounding media. To test the effects of these extracts on bacterial growth, solutions were created consisting of 33.3% of one of the extracts, 26.7% distilled water, 33.3% of homemade nutrient broth, and 6.7% of either an overnight *E. coli* or *E. aerogenes* culture. Nearly identical control solutions were also created, substituting DS for equal amount of distilled water and LS for an equal quantity of homemade nutrient broth. The cultures were then incubated at 37°C under aerobic conditions for 23.8 hours. The cultures' optical densities at 595 nm were measured initially, in 2.5 hours, 4.8 hours, and 23.8 hours. We subtracted the initial OD 595 readings from those at 2.5, 4.8, and 23.8 hours to calculate growth values. Data were analyzed using SigmaPlot to determine the standard error of the mean and P-values through two tailed unpaired t-tests.

Storage

E. coli death quorum sensors were isolated according to the procedure above and subsequently stored either at 37°C, 25°C, 4°C, or -20°C for 10 days. Experimental solutions consisted of 33.3% *E. coli* clarified death phase supernatant stored in each of the listed conditions, 6.7% overnight cultures of *E. coli,* 33.3% of nutrient broth, and 26.7% of distilled water. A control solution was created, substituting the *E. coli* clarified death phase supernatant for an equal quantity of distilled water. The cultures were then incubated at 37°C under aerobic conditions for 23.8 hours. The cultures' optical densities were measured initially, after 2.8 hours, 6.2 hours, and 23.8 hours.

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