Suppression of *E. coli* Biofilm Formation with Garlic Extract

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Abstract

Antibiotics have revolutionized human medicine by providing powerful tools to combat bacterial illnesses, but the rise of antibiotic resistance threatens to render them useless. One type of antibiotic resistance, called a biofilm, might be inhibited by quorum sensing inhibitors, which block bacterial communication. Because quorum sensing inhibitors do not rely on killing bacteria, it may take longer for bacteria to develop resistance against them. Garlic extract, which contains a quorum sensing inhibitor, is tested in this research for efficacy against the biofilm formation of *E. coli*, specifically the K-12 MG1655 strain, because *E. coli* is a major cause of food-borne illness. If garlic extract proves effective, it could help prevent *E. coli* biofilms from surviving cleaning procedures, protecting against ingestion. After testing a variety of concentrations, this research finds that garlic extract at a concentration of 16 µL / mL likely promotes biofilm formation but that it inhibits formation at a concentration of 64 µL / mL. However, the data are ultimately inconclusive. Thus, while additional research is required, garlic extract could prove valuable in preventing food-borne illness.

Introduction

While antibiotics, perhaps more than any invention of the twentieth century, have dramatically improved public health, those gains are now in jeopardy in the face of antibiotic-resistant strains of lethal bacterial diseases. From MRSA to salmonella to *E. coli*, resistant strains are expensive if not impossible to treat, so they pose a grave threat to public health.[1] The biofilm is one particularly dangerous form of antibiotic resistance. Biofilms are colonies of bacteria that anchor themselves to biotic or abiotic

[1] United States. Congress. House. Committee on Energy and Commerce. Subcommittee on Health, 2012

surfaces and then secrete a matrix of polysaccharides, proteins, and DNA around themselves.[2] This matrix protects the bacteria inside from a variety of environmental stressors, including host immune systems and antibiotics.[3] Additionally, once the biofilms of some strains, including salmonella, are established, they can withstand being soaked in disinfectants like sodium hydroxide.[4] This is particularly dangerous because salmonella is a common food-borne illness. Biofilms can also form on produce and survive cleaning procedures to infect whomever eats the contaminated produce.[5] Finally, biofilms can form inside the body. In particular, *P. aeruginosa* forms biofilms in the lungs of cystic fibrosis patients[6] despite high doses of antibiotics.[7]

Garlic extract has the potential to fight biofilms better than current antibiotics because it contains the compound ajoene, which inhibits quorum sensing. Quorum sensing is a type of bacterial communication that allows individual bacteria to measure the concentration of other bacteria around themselves. As part of this process, each bacterium releases signaling molecules, called autoinducers,[8] into its environment, so the concentration of signaling molecules is related to the concentration of bacteria.[9] This communication is critical for biofilm formation because high concentrations of autoinducers trigger behavioral shifts in bacteria, including the start of biofilm formation. Quorum sensing inhibitors, molecules that block quorum sensing, can prevent biofilm formation and reduce the virulence of the bacteria.[10] Additionally, because quorum sensing inhibition makes bacteria less dangerous instead of killing them, there is a weaker selective force favoring any resistant bacteria.[11] Therefore, it puts less evolutionary pressure on bacteria, so bacteria are less likely to develop resistance against it. Ajoene is an effective quorum sensing inhibitor and reduces biofilm formation by *P. aeruginosa* in mammalian systems,[12] so garlic extract is a promising candidate for future antibiotic treatments.

While previous research has examined quorum sensing inhibitors, including ajoene, as antibiotic treatment options, there are still gaps in science's understanding where additional research is needed. Past work has largely focussed on biofilms in the body, so *P. aeruginosa*, biofilms in cystic fibrosis patients' lungs, and biofilms on biomedical implants have been examined extensively. Additionally, much of the past research on

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2 Absalon, Van Dellen, & Watnick, 2011
3 Redelman, Chakravarty, & Anderson, 2014
4 Corcoran et al., 2014
5 Annous, Fratamico, & Smith, 2009
6 Jakobsen et al., 2012
7 Redelman, Chakravarty, & Anderson, 2014
8 O'Loughlin et al., 2013
9 How bacteria "talk," 2009
10 O'Loughlin et al., 2013
11 Tay & Yew, 2013
12 Jakobsen et al., 2012

ajoene has used extracted or synthesized ajoene. However, little or no research has been done on the effectiveness of raw garlic extract for inhibiting biofilms formed on produce. Research has been done on crude garlic extract as a quorum sensing inhibitor and as an inhibitor of *P. aeruginosa* biofilms,[13] but not as an inhibitor of *E. coli* biofilms. As a first step towards filling that gap, this research examines the impact of garlic extract on the biofilm formation of *E. coli*, a principal culprit in food-borne illness. Thus, this research seeks to answer the question, "Does garlic extract suppress biofilm formation by *E. coli* K-12 MG1655?"

In order to isolate the changes in biofilm formation due to the concentration of garlic extract, a number of confounding variables have to be controlled. Contamination has to be prevented as much as possible and detected if it does occur. The biofilm attachment surface must be kept identical across samples, and the growth in each sample needs to be approximately equal, excluding inhibitory effects by garlic extract. Changes to the cuvette by the compounds used also has to be prevented, the growth media must be constant, and errors in concentrations due to imprecise volume measurements or non-uniform solutions need to be minimized.

To more effectively combat antibiotic resistance, the healthcare and food industries need better antibiotic compounds. However, before those can be developed, let alone used, they have to be identified and tested. This research will contribute evidence of garlic extract's efficacy or inefficacy to that search. That evidence may help future researchers direct their resources more efficiently and expedite the creation of more effective antibiotics. Furthermore, this work will contribute another perspective to a broader conversation, perhaps further reducing the subjectivity, and therefore increasing the reliability, of that conversation's conclusions.

Hypothesis

For this experiment, it is hypothesized that if the right concentration of garlic extract is added to a liquid culture of *E. coli* K-12 MG1655, biofilm formation will be reduced. This is based on research showing that ajoene reduces the amount of biofilm formed by *P. aeruginosa* in mammalian systems by inhibiting quorum sensing[14] and research showing that crude garlic extract reduces quorum sensing.[15] Since *E. coli* uses quorum sensing, it is believed that garlic extract will also reduce biofilm formation by *E. coli*.

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13 Rasmussen et al., 2005
14 Jakobsen et al., 2012
15 Rasmussen et al., 2005

Methods

For the stock colonies of bacteria used in this research, dehydrated *E. coli* K-12 MG1655 was ordered. The dehydrated bacteria were revived by adding liquid LB media to the filter paper and then spreading that inoculated liquid on a lysogeny broth (LB)[16] agar petri dish with a sterile, plastic inoculating loop. The LB agar plates were made by mixing powdered LB agar with deionized water in a ratio of 32.00 g per 1 L, per the manufacturer's instructions. That mixture was stirred until it dissolved as much as possible, and then it was sterilized. Sterilization was performed with a pressure cooker on for at least 10. mins. after its valve sealed. In the case of liquids, the cooker was slowly depressurized either by letting it cool or by depressing the valve no faster than once every 2 sec. The agar was then poured into petri dishes to make the plates for bacteria to grow on. Cultures were cycled every 2-4 wks. Each time, sterile wooden inoculating sticks were used to inoculate a new petri dish from the youngest existing colony.

Liquid cultures of bacteria were grown in liquid LB media. This was made by mixing the powdered form of the media with deionized water in a ratio of 20.00 g per 1 L, per the manufacturer's instructions, and then sterilizing the mixture.

On the first day of each experiment performed for this research, a culture and a negative control were made in liquid LB media. Two 125 mL Erlenmeyer flasks, each with 10.0 mL of media, were sterilized with the media inside. Then, one was inoculated with a sterile, wooden inoculating stick and the other was left as a negative control. Both were incubated at 37°C for 48 hrs.

After 48 hrs. of incubation, on the 3rd day of each experiment, the culture was diluted by a factor of 100. Some of that diluted culture was split among 8 sterile glass test tubes. To 4 of those tubes, differing amounts of garlic extract were added to test different concentrations. To the other 4 tubes, 95% ethanol was added in the same amounts as the garlic extract. This functioned as the vehicle control as the garlic extract was dissolved in 95% ethanol. Once those mixtures were made up, each one was divided among five replicates. Each replicate contained 1 mL of solution in a plastic sterile test tube. There were also 5 replicates of the diluted culture and 5 of the negative control from the Erlenmeyer flask. All the replicates were incubated at 37°C for 24 hrs.

[16] Bertani, 2004

The next day, day 4, all of the replicate test tubes were emptied and rinsed 4 times with deionized water to eliminate any unattached bacteria. To rinse a tube, it was filled with water and then emptied. To each replicate tube, 2 mL of 0.100% crystal violet were added to stain the biofilm. After incubating for 30.0 mins. at room temperature, the replicates were emptied and rinsed 4 times to remove excess stain, revealing the stained biofilm as a purple ring where the interface between the culture and the air used to be. The tubes were left to dry upside-down overnight.

On the 5th and final day of the experiment, each replicate was banged on the table-top to shake out excess water, and each was filled with 3 mL of 95% ethanol to dissolve the stained biofilm. After a 15.0 min., or however long it took for the rings to dissolve, incubation at room temperature, a glass cuvette was filled with the solution and read with a UV Spectrophotometer at OD600 (600.0 nm). The cuvette was rinsed with 95% ethanol between each reading. Liquid was transferred using disposable plastic transfer pipets, with a new one used for each replicate.

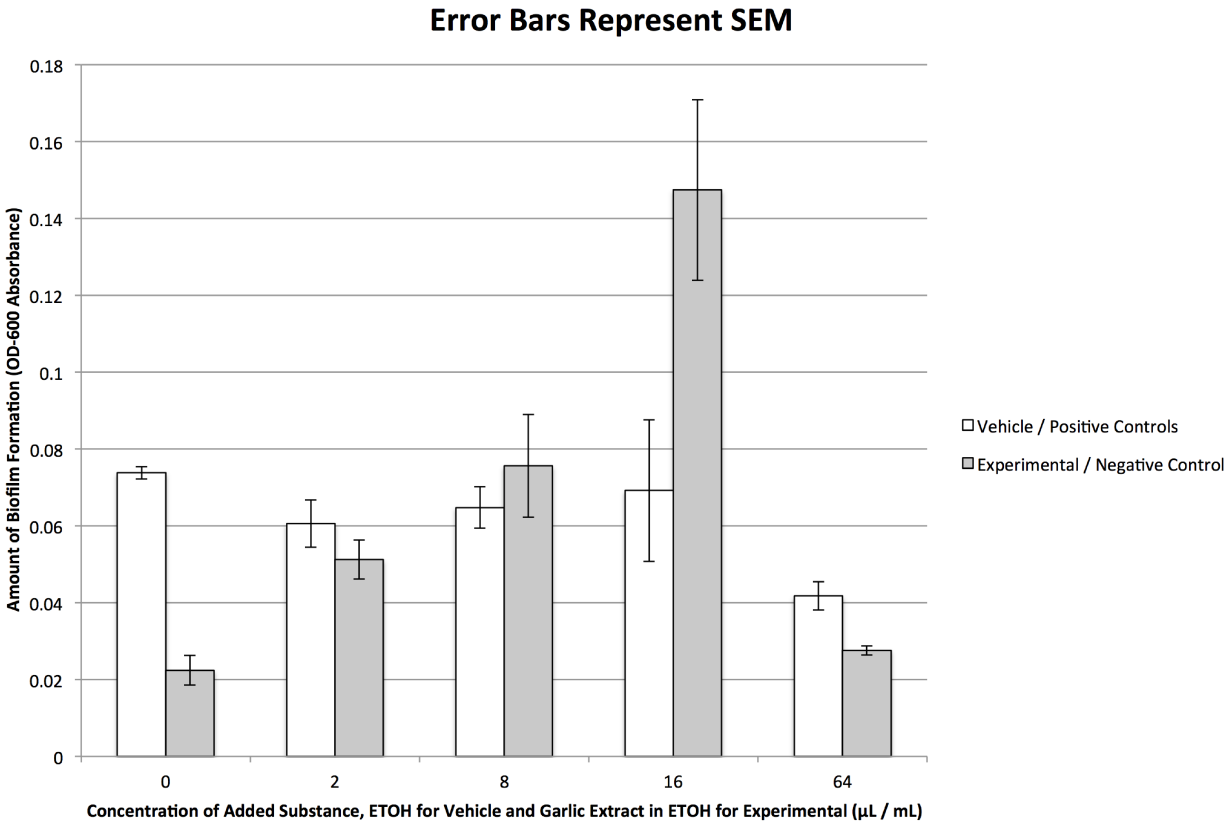
Overall, this protocol measured the amount of biofilm formed, the dependent variable, using crystal violet and UV spectroscopy when varying concentrations of garlic extract, the independent variable, were added.

To account for changes introduced by the ethanol the garlic extract was dissolved in, a set of vehicle controls, each one of which had the same concentration of ethanol as an experimental had garlic extract, were kept alongside the experimental samples. Without this measure, it would have been impossible to tell whether the differences measured were due to garlic extract or ethanol. In addition, to reduce contamination, media was sterilized after transfer when possible and the openings of tubes and bottles were flamed before and after adding or removing liquid. In case contaminant organisms did slip through, negative controls were treated just like the other samples and monitored for growth. This prevented any organisms from growing and changing measured growth through competition or biofilm formation without detection. Furthermore, to reduce variations in concentration, heterogeneous mixtures were vortexed or swirled before any liquid was removed and all replicates of a sample came from the same mixture. These measures helped prevent variations in concentrations obscuring actual results. Finally, a glass cuvette was used to avoid any changes to the cuvette surface by the ethanol or crystal violet, and all tubes were the same product from the same manufacturer to further reduce variation.

Results

Trial 1

Graph 1: Impact of Garlic Extract on Biofilm Formation (Trial 1)



In the graph above, experimental (gray) and vehicle control (white) samples are grouped together at each concentration tested (2.0, 8.0, 16, and 64 µL / mL). The bars at 0 µL / mL represent the negative (gray) and positive (white) controls.

In Graph 1, little difference is apparent at concentrations of 2.0 and 8.0 µL / mL. However, at 16 µL / mL, the experimental samples showed much greater biofilm

formation than the vehicle control. Conversely, at 64 µL / mL, there was less biofilm formation in the experimental than in the vehicle control. Error bars suggest that both of these differences are significant, though T-Tests will be used to know for sure.

Table 1: Trial 1 Statistical Analysis

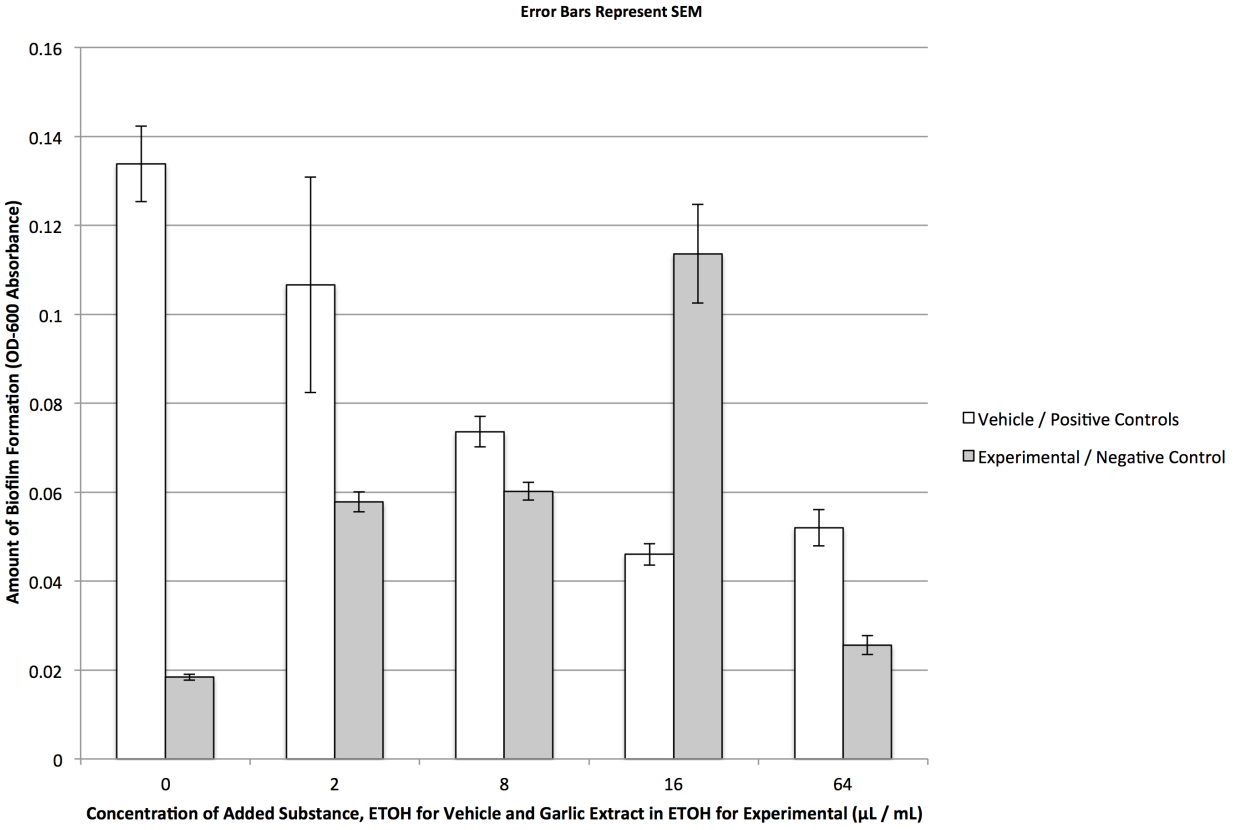
T-Test Table	NC	PC	E1	E2	E3	E4	V1	V2	V3	V4
NC	1.000									
PC	0.000	1.000								
E1	0.002	0.009	1.000							
E2	0.014	0.900	0.148	1.000						
E3	0.005	0.035	0.014	0.036	1.000					
E4	0.256	0.000	0.008	0.023	0.007	1.000				
V1	0.001	0.098	0.274	0.350	0.019	0.005	1.000			
V2	0.000	0.173	0.104	0.486	0.023	0.002	0.621	1.000		
V3	0.063	0.816	0.393	0.787	0.032	0.087	0.677	0.828	1.000	
V4	0.007	0.000	0.177	0.063	0.010	0.015	0.036	0.009	0.214	1.000

The table above can be used to find the results of a two-tailed T-Test assuming unequal variance between any two samples. Any given cell containing a number holds the p value from the T-Test between the samples indicated in the headers for its row and column. “E” indicates an experimental sample while “V” indicates a vehicle control one. The numbers following an “E” or a “V” indicate successively higher concentrations (i.e. “E3” indicates the experimental sample with a concentration of 16 µL / mL). Gray cells indicate values less than 0.05. Cells with borders are those that compare an experimental sample with the associated vehicle control. Half of the table is blank because it would be a mirror image of the completed half.

The p values shown in the boxed sections of Table 1 reveal that the differences apparent in Figure 1 (at 16 and 64 µL / mL) are indeed statistically significant. However, because the samples in this case were replicates, they were not independent samples. Thus, this does not demonstrate overall statistical significance

Trial 2[17]

Graph 2: Impact of Garlic Extract on Biofilm Formation (Trial 2)



The graph above has the same format and notation as that in Trial 1.

In Graph 2, little difference is apparent at a concentration 8.0 µL / mL. However, at 16 µL / mL, the experimental samples showed much greater biofilm formation than the vehicle control. Conversely, at 64 µL / mL and 2.0 µL / mL, there was less biofilm formation in the experimental samples than in the vehicle controls. Error bars suggest that both of these differences are significant, though T-Tests will be used to know for sure.

[17] Note that another trial was performed between trials 1 and 2, but because of human errors in carrying out the procedure, it was deemed flawed and the results were discarded.

Table 2: Trial 2 Statistical Analysis

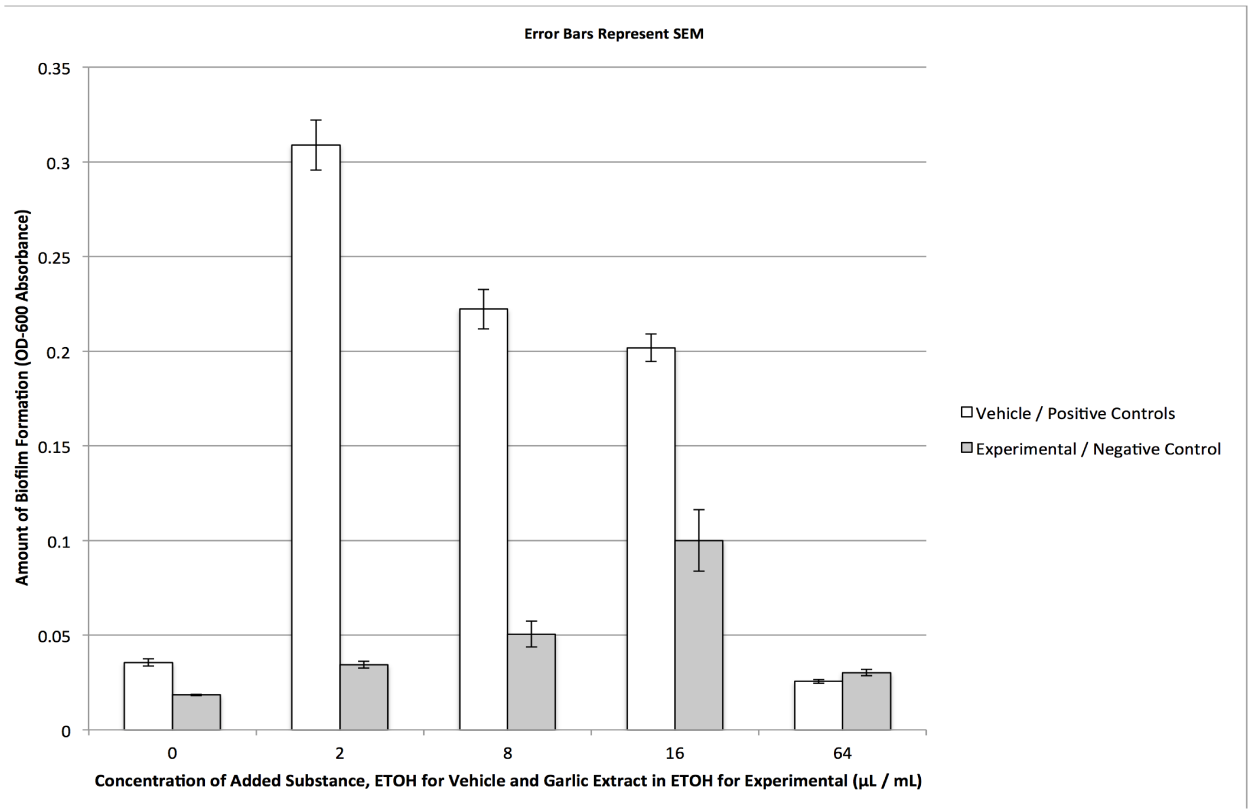
T-Test Table	NC	PC	E1	E2	E3	E4	V1	V2	V3	V4
NC	1.000									
PC	0.000	1.000								
E1	0.000	0.001	1.000							
E2	0.000	0.001	0.447	1.000						
E3	0.001	0.188	0.006	0.008	1.000					
E4	0.024	0.000	0.000	0.000	0.001	1.000				
V1	0.022	0.338	0.114	0.128	0.802	0.028	1.000			
V2	0.000	0.001	0.007	0.014	0.020	0.000	0.246	1.000		
V3	0.000	0.000	0.007	0.002	0.003	0.000	0.066	0.000	1.000	
V4	0.001	0.000	0.257	0.122	0.003	0.001	0.086	0.004	0.248	1.000

The table above has the same format and notation as that in Trial 1.

The p values shown in the boxed sections of Table 2 reveal that the differences apparent in Figure 2 (at 8. 0, 16 and 64 µL / mL) are statistically significant. However, because the samples in this case were replicates, they were not independent samples. Thus, this evidence does not demonstrate overall statistical significance.

Trial 3

Graph 3: Impact of Garlic Extract on Biofilm Formation (Trial 3)



The graph above has the same format and notation as that in Trial 1.

In Graph 3, little difference is apparent at a concentration of 64 µL / mL, though the experimental samples may have shown slightly more growth. However, at 2.0, 8.0, and 16 µL / mL, the experimental samples showed much less biofilm formation than the associated vehicle controls. Error bars suggest that these three differences are significant, though T-Tests will be used to know for sure. This sharply contrasts the results of Trials 1 and 2.

Table 3: Trial 3 Statistical Analysis

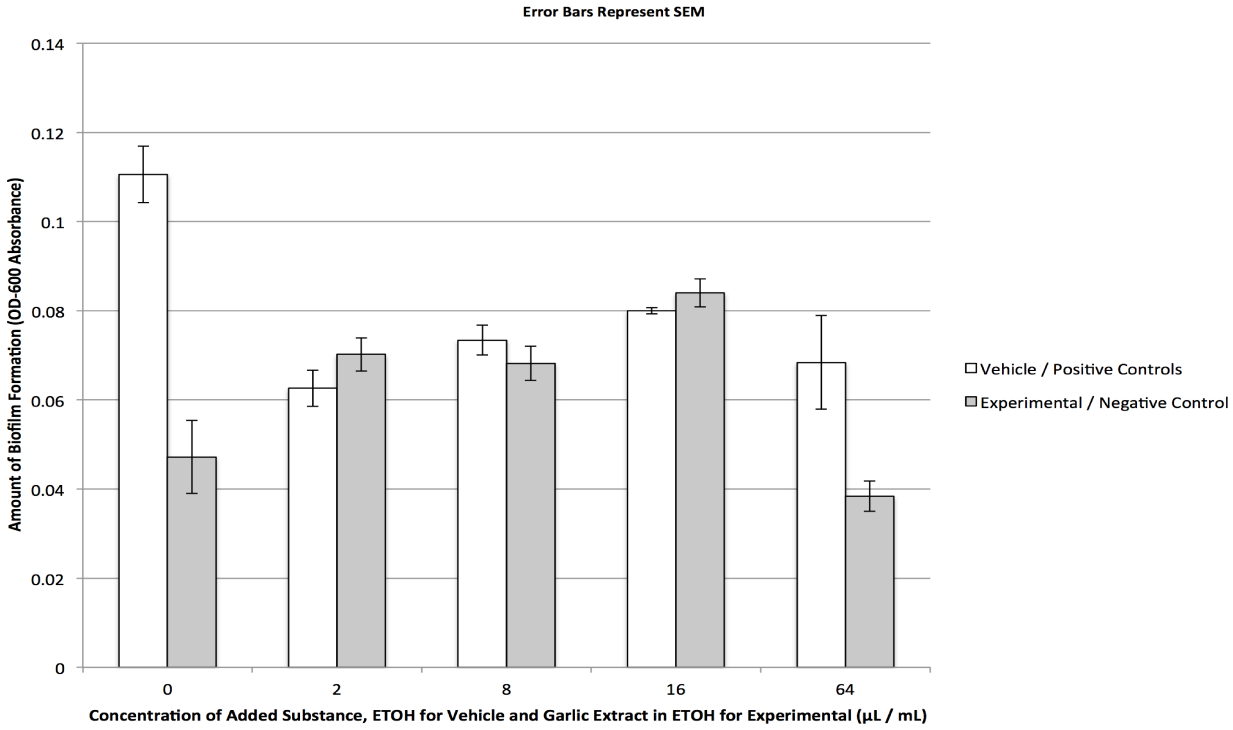
T-Test Table	NC	PC	E1	E2	E3	E4	V1	V2	V3	V4
NC	1.000									
PC	0.001	1.000								
E1	0.001	0.663	1.000							
E2	0.009	0.093	0.076	1.000						
E3	0.007	0.016	0.015	0.035	1.000					
E4	0.001	0.068	0.120	0.039	0.012	1.000				
V1	0.000	0.000	0.000	0.000	0.000	0.000	1.000			
V2	0.000	0.000	0.000	0.000	0.000	0.000	0.001	1.000		
V3	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.153	1.000	
V4	0.001	0.004	0.004	0.021	0.010	0.048	0.000	0.000	0.000	1.000

The table above has the same format and notation as that in Trial 1.

The p values shown in the boxed sections of Table 3 reveal that the differences apparent in Figure 3 (at all concentrations) are indeed statistically significant. However, because the samples in this case were replicates, they were not independent samples. Therefore, this does not demonstrate overall statistical significance.

Trial 4

Graph 4: Impact of Garlic Extract on Biofilm Formation (Trial 4)



The graph above has the same format and notation as that in Trial 1.

In Graph 4, little difference is apparent at any concentration, except for at 64 µL / mL. There, there was more biofilm formation in the vehicle control. Error bars suggest that this difference is significant, though T-Tests will be used to know for sure. At 2.0 and 16 µL / mL, it appears there was slightly more biofilm formation in the experimental samples, with the reverse true for 8.0 µL / mL. These results are far less clear than those of the first three trials, though the difference at 64 µL / mL matches that in Trials 1 and 2.

Table 4: Trial 4 Statistical Analysis

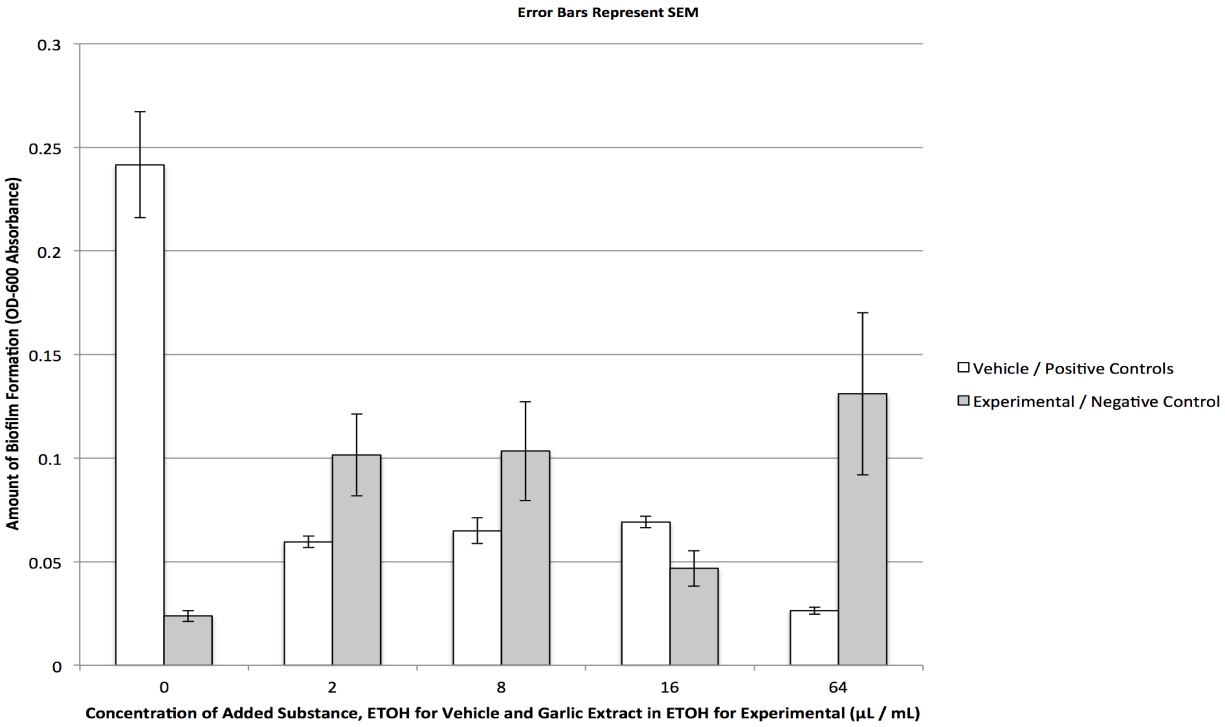
T-Test Table	NC	PC	E1	E2	E3	E4	V1	V2	V3	V4
NC	1.000									
PC	0.000	1.000								
E1	0.045	0.001	1.000							
E2	0.061	0.001	0.718	1.000						
E3	0.008	0.010	0.023	0.013	1.000					
E4	0.362	0.000	0.000	0.000	0.000	1.000				
V1	0.143	0.000	0.205	0.345	0.004	0.002	1.000			
V2	0.029	0.002	0.539	0.336	0.049	0.000	0.075	1.000		
V3	0.016	0.008	0.057	0.036	0.275	0.000	0.012	0.118	1.000	
V4	0.152	0.012	0.878	0.986	0.217	0.043	0.627	0.669	0.331	1.000

The table above has the same format and notation as that in Trial 1.

The p values shown in the boxed sections of Table 4 reveal that the difference apparent in Figure 4 at a concentration of 64 µL / mL is indeed statistically significant. However, because the samples in this case were replicates, they were not independent samples. Therefore, this does not demonstrate overall statistical significance.

Trial 5

Graph 5: Impact of Garlic Extract on Biofilm Formation (Trial 5)



The graph above has the same format and notation as that in Trial 1.

In Graph 5, there seems to have been more biofilm formation in experimental groups at concentrations of 2.0, 8.0, and 64 µL / mL. On the other hand, at 16 µL / mL, there appears to be less growth in the experimental samples. Error bars suggest that the three differences at 2.0 and 64 µL / mL may be significant, though T-Tests will be used to know for sure. The results at 2.0 µL / mL contrast those from Trials 2 and 3, and those at 64 µL / mL contradict those from Trials 1, 2, and 4. Some trials were excluded from those comparisons due to a lack of substantial difference between experimental and vehicle control samples.

Table 5: Trial 5 Statistical Analysis

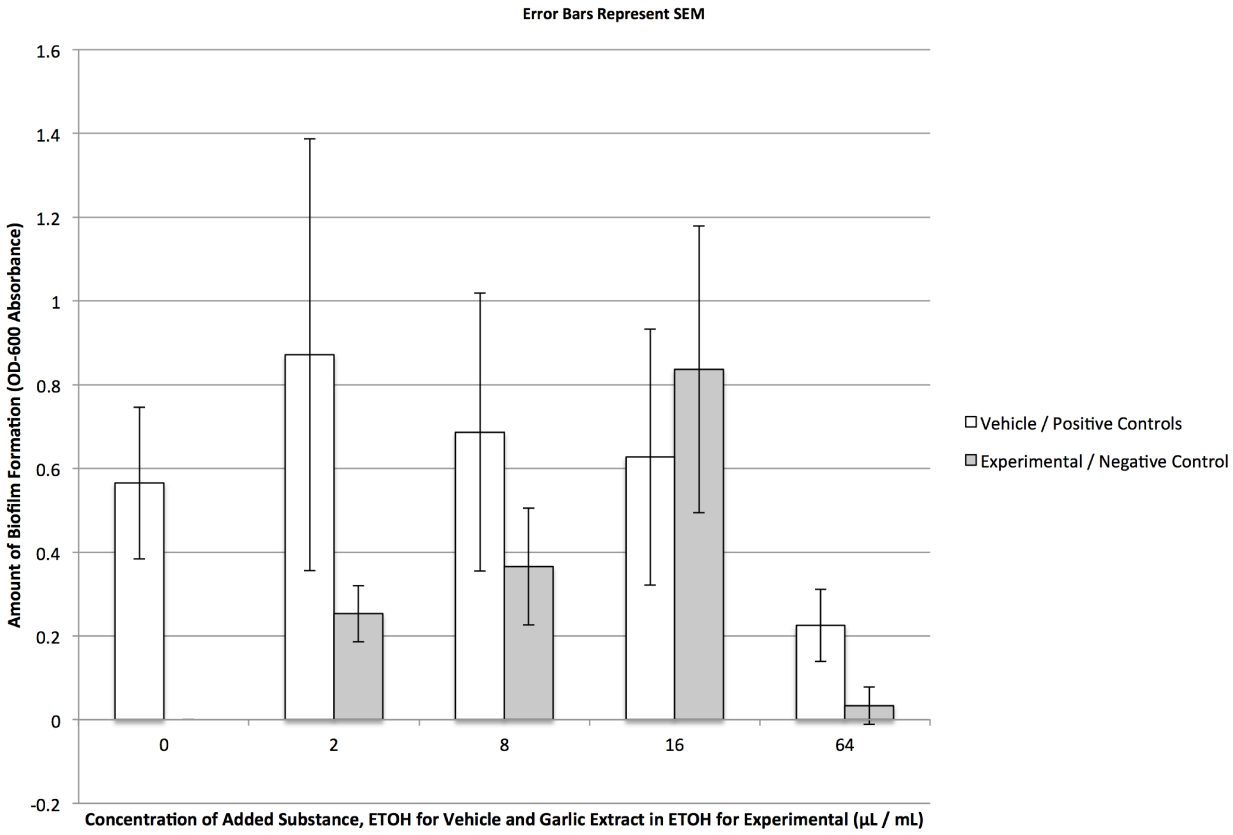
T-Test Table	NC	PC	E1	E2	E3	E4	V1	V2	V3	V4
NC	1.000									
PC	0.001	1.000								
E1	0.016	0.003	1.000							
E2	0.028	0.004	0.955	1.000						
E3	0.053	0.001	0.048	0.076	1.000					
E4	0.052	0.050	0.528	0.567	0.097	1.000				
V1	0.000	0.002	0.100	0.140	0.218	0.142	1.000			
V2	0.001	0.002	0.140	0.186	0.130	0.168	0.467	1.000		
V3	0.000	0.002	0.177	0.226	0.058	0.190	0.041	0.566	1.000	
V4	0.432	0.001	0.019	0.032	0.076	0.056	0.000	0.003	0.000	1.000

The table above has the same format and notation as that in Trial 1.

These p-values indicate that none of the differences observed in Figure 5 are statistically significant if replicates are taken as independent samples.

Overall

Graph 6: Impact of Garlic Extract on Biofilm Formation (All Trials)



The graph above has the same format and notation as that in Trial 1.

Graph 6, which combines data from all five trials, reveals that the differences between experimental and vehicle control samples are small compared to the variations represented by error bars. The data from the five trials were normalized before combined. Normalization was done by subtracting from the average readings in each trial the average value from the negative control and then dividing by the average value for the positive control.

Table 6: Statistical Analysis over Both Trials

T-Test Table	NC	PC	E1	E2	E3	E4	V1	V2	V3	V4
NC	1.000									
PC	0.010	1.000								
E1	0.005	0.056	1.000							
E2	0.019	0.147	0.416	1.000						
E3	0.065	0.861	0.199	0.306	1.000					
E4	0.257	0.017	0.176	0.095	0.105	1.000				
V1	0.125	0.822	0.288	0.389	0.936	0.180	1.000			
V2	0.076	0.868	0.269	0.430	0.782	0.132	0.758	1.000		
V3	0.073	0.733	0.293	0.487	0.683	0.135	0.681	0.897	1.000	
V4	0.064	0.028	0.356	0.176	0.139	0.572	0.222	0.181	0.190	1.000

The table above has the same format and notation as that in Trial 1.

The p values shown in the boxed sections of Table 3 reveal that the differences are not statistically significant.

Conclusion

Overall, these results do not conclusively answer the question of whether garlic extract suppresses biofilm formation by *E. coli* K-12 MG1655. The lack of statistically significant differences means that they fail to support the hypothesis, but that same uncertainty also means that they fail to refute it. The lack of significance could stem from methodological problems, so significant results were expected but not guaranteed. This means that the hypothesis could still be correct.

Despite this uncertainty, they do suggest that garlic extract at 64 µL / mL, might reduce biofilm formation. Surprisingly, they also indicate that at a concentration of 16 µL / mL, garlic extract may promote biofilm formation.

Across the trials that were conducted, which were conducted on different weeks and are therefore considered independent, the samples with 64 µL / mL garlic extract, which was dissolved in 95% ethanol, generally (in trials 1, 2, and 4) showed less biofilm formation than those with an equal amount of 95% ethanol. This can be seen in Graphs 1 -5 as the differences in white and gray bars at 64 µL / mL, and it suggests that the garlic extract may have been effective in inhibiting biofilm formation.

On the other hand, many trials (trials 1, 2, and 4) also showed an increase in biofilm formation at 16 µL / mL in the experimental samples compared to the vehicle controls. This is evident in Graphs 1-5 as the differences between white and gray bars at 16 µL / mL, and it suggests that garlic extract may have promoted biofilm growth at this concentration. However, for both of these suggestions, there was no consensus across all trials.

Additionally, T-Tests on the replicates from the trials (shown in the boxed sections of Tables 1-5) show that the differences at 16 and 64 µL / mL are generally statistically significant. However, because the samples used in a T-Test need to be independent, unlike the replicates used, the p values only indicate that the difference measured is likely not due to random variation within the trial. Variations affecting the entire group of samples could still be at play, which is why multiple trials were performed. Many trials found the same results, which suggests that the differences in the graphs reflect differences in the broader population, but T-Tests using the trials as independent samples (shown in the boxed sections of Table 6) indicate that the differences were not statistically significant. However, the similarities among trials suggest that a methodology with less variation might reveal significant differences.

While these results reveal no definite link between garlic extract concentration and biofilm formation, they do show that such a link may still exist. That suggestion marks garlic extract as a potential candidate for new antibiotics designed to thwart antibiotic resistance. Future research on garlic extract may produce powerful new antibiotic compounds to help treat an increasingly dangerous epidemic of antibiotic-resistant bacteria. Additionally, these results warn that if used improperly, such as at too low of a dosage, garlic extract or its derivative medicines could help bacteria form the very antibiotic-resistant biofilms the medication is designed to prevent. This warning may help dissuade patients from skipping their medications and avoid life-threatening resurgences of diseases. Ultimately, however, these results are inconclusive and fail to either support or refute the initial hypothesis.

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Appendix A: Raw Data

The tables below present the raw measurements collected during the two trials, along with some calculations. The five replicates are displayed in columns, followed by the mean, the 95% confidence interval, the standard deviation, and the standard error of the mean that are calculated from those five replicates. Note that only the standard error of the mean (SEM) and the mean are displayed in the graphs. The experimental and vehicle control samples are grouped under a corresponding label, and the row headers indicate the concentrations of ethanol or garlic extract added.

Table 7: Trial 1 Raw Data

Raw Data	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Mean	95% Conf.	Standard Deviation	Standard Error of the Mean
Negative Control	0.036	0.023	0.015	0.015	0.023	0.022	0.0107	0.0086	0.0038
Positive Control	0.079	0.076	0.071	0.072	0.071	0.074	0.0044	0.0036	0.0016
Experimental (µL / mL)									
2.0	0.071	0.049	0.048	0.046	0.042	0.051	0.0141	0.0114	0.0051
8.0	0.121	0.091	0.061	0.054	0.051	0.076	0.0372	0.0299	0.0134
16	0.233	0.143	0.150	0.116	0.095	0.147	0.0654	0.0526	0.0235
64	0.026	0.025	0.027	0.032	0.028	0.028	0.0034	0.0027	0.0012
Vehicle Control (µL / mL)									
2.0	0.076	0.069	0.062	0.056	0.040	0.061	0.0171	0.0137	0.0061
8.0	0.070	0.068	0.079	0.060	0.047	0.065	0.0149	0.0120	0.0054
16	0.129	0.096	0.042	0.039	0.040	0.069	0.0512	0.0412	0.0184
64	0.029	0.040	0.049	0.049	0.042	0.042	0.0102	0.0082	0.0037

Table 8: Trial 2 Raw Data

Raw Data	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Mean	95% Conf.	Standard Deviation	Standard Error of the Mean
Negative Control	0.019	0.020	0.019	0.018	0.016	0.018	0.0019	0.0015	0.0007
Positive Control	0.115	0.136	0.136	0.163	0.119	0.134	0.0235	0.0189	0.0085
Experimental (µL / mL)									
2.0	0.065	0.057	0.051	0.058	0.058	0.058	0.0062	0.0050	0.0022
8.0	0.060	0.064	0.064	0.060	0.053	0.060	0.0056	0.0045	0.0020
16	0.156	0.092	0.112	0.103	0.105	0.114	0.0307	0.0248	0.0111
64	0.033	0.024	0.025	0.020	0.026	0.026	0.0059	0.0047	0.0021
Vehicle Control (µL / mL)									
2.0	0.182	0.133	0.108	0.055	0.055	0.107	0.0672	0.0541	0.0242
8.0	0.067	0.068	0.078	0.085	0.070	0.074	0.0096	0.0077	0.0034
16	0.054	0.045	0.039	0.047	0.045	0.046	0.0067	0.0054	0.0024
64	0.040	0.061	0.051	0.047	0.061	0.052	0.0113	0.0091	0.0041

Table 9: Trial 3 Raw Data

Raw Data	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Mean	95% Conf.	Standard Deviation	Standard Error of the Mean
Negative Control	0.018	0.019	0.019	0.017	0.019	0.018	0.0011	0.0009	0.0004
Positive Control	0.039	0.037	0.030	0.040	0.032	0.036	0.0055	0.0044	0.0020
Experimental (µL / mL)									
2.0	0.041	0.035	0.033	0.031	0.032	0.034	0.0049	0.0040	0.0018
8.0	0.074	0.056	0.048	0.035	0.040	0.051	0.0190	0.0153	0.0069
16	0.160	0.106	0.084	0.083	0.067	0.100	0.0451	0.0363	0.0162
64	0.025	0.028	0.034	0.032	0.032	0.030	0.0045	0.0036	0.0016
Vehicle Control (µL / mL)									
2.0	0.265	0.300	0.319	0.346	0.314	0.309	0.0368	0.0296	0.0132
8.0	0.198	0.202	0.219	0.251	0.241	0.222	0.0290	0.0234	0.0105
16	0.176	0.195	0.212	0.213	0.213	0.202	0.0203	0.0163	0.0073
64	0.022	0.026	0.026	0.028	0.026	0.026	0.0027	0.0022	0.0010

Table 10: Trial 4 Raw Data

Raw Data	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Mean	95% Conf.	Standard Deviation	Standard Error of the Mean
Negative Control	0.059	0.069	0.051	0.027	0.030	0.047	0.0227	0.0183	0.0082
Positive Control	0.121	0.119	0.122	0.091	0.100	0.111	0.0176	0.0142	0.0063
Experimental (µL / mL)									
2.0	0.058	0.068	0.078	0.078	0.069	0.070	0.0103	0.0083	0.0037
8.0	0.058	0.060	0.076	0.072	0.075	0.068	0.0106	0.0086	0.0038
16	0.095	0.085	0.081	0.076	0.083	0.084	0.0087	0.0070	0.0031
64	0.032	0.051	0.038	0.033	0.038	0.038	0.0094	0.0076	0.0034
Vehicle Control (µL / mL)									
2.0	0.061	0.052	0.057	0.068	0.075	0.063	0.0113	0.0091	0.0041
8.0	0.073	0.075	0.078	0.080	0.061	0.073	0.0092	0.0074	0.0033
16	0.080	0.079	0.082	0.081	0.078	0.080	0.0020	0.0016	0.0007
64	0.059	0.038	0.065	0.101	0.079	0.068	0.0291	0.0234	0.0105

Table 11: Trial 5 Raw Data

Raw Data	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Mean	95% Conf.	Standard Deviation	Standard Error of the Mean
Negative Control	0.033	0.019	0.020	0.021	0.026	0.024	0.0072	0.0058	0.0026
Positive Control	0.217	0.206	0.238	0.206	0.341	0.242	0.0709	0.0571	0.0255
Experimental (µL / mL)									
2.0	0.062	0.108	0.083	0.080	0.175	0.102	0.0549	0.0442	0.0198
8.0	0.072	0.070	0.066	0.118	0.191	0.103	0.0662	0.0534	0.0239
16	0.081	0.039	0.040	0.035	0.039	0.047	0.0239	0.0192	0.0086
64	0.060	0.058	0.155	0.112	0.270	0.131	0.1087	0.0875	0.0391
Vehicle Control (µL / mL)									
2.0	0.066	0.051	0.055	0.062	0.064	0.060	0.0079	0.0063	0.0028
8.0	0.086	0.051	0.053	0.066	0.069	0.065	0.0175	0.0141	0.0063
16	0.068	0.074	0.072	0.073	0.059	0.069	0.0076	0.0061	0.0027
64	0.033	0.025	0.026	0.023	0.025	0.026	0.0048	0.0038	0.0017

Table 12: Raw Data for Combining All Trials

Raw Data (µL / mL)	Exp 16 Raw Mean	Exp 18 Raw Mean	Exp 19 Raw Mean	Exp 20 Raw Mean	Exp 21 Raw Mean	Exp 16 Norm. Mean	Exp 18 Norm. Mean	Exp 19 Norm. Mean	Exp 20 Norm. Mean	Exp 21 Norm. Mean	Mean	95% Conf.	Standard Deviation	Standard Error of Mean
Negative Control	0.022	0.018	0.0184	0.0472	0.0238	0.000	0.000	0.000	0.000	0.000	0.000	0.0000	0.0000	0.0000
Positive Control	0.074	0.134	0.0356	0.1106	0.2416	0.696	0.862	0.129	0.573	0.901	0.565	0.7807	0.3143	0.1815
Experimental														
2	0.051	0.058	0.0344	0.0702	0.1016	0.390	0.294	0.120	0.208	0.322	0.253	0.2882	0.1160	0.0670
8	0.076	0.060	0.0506	0.0682	0.1034	0.721	0.312	0.241	0.190	0.329	0.366	0.6009	0.2419	0.1397
16	0.147	0.114	0.1	0.084	0.0468	1.694	0.712	0.610	0.333	0.095	0.837	1.4736	0.5932	0.3425
64	0.028	0.026	0.0302	0.0384	0.131	0.070	0.054	0.088	-0.080	0.444	0.033	0.1900	0.0765	0.0442
Vehicle Control														
2	0.061	0.107	0.3088	0.0626	0.0596	0.518	0.659	2.170	0.139	0.148	0.872	2.2189	0.8932	0.5157
8	0.065	0.074	0.2222	0.0734	0.065	0.575	0.413	1.523	0.237	0.171	0.687	1.4268	0.5744	0.3316
16	0.069	0.046	0.2018	0.08	0.0692	0.634	0.206	1.371	0.297	0.188	0.627	1.3140	0.5289	0.3054
64	0.042	0.052	0.0256	0.0684	0.0264	0.263	0.401	0.043	0.192	0.011	0.225	0.3700	0.1489	0.0860

The table above presents, in the first five columns, the average values for each sample from the five trials conducted. The next five columns contain the normalized forms of the first five. Normalization was achieved by subtracting the values from the negative control and expressing the result as a fraction of the positive control readings.

Appendix B: Figures

The figures below document both the growth and the staining visible in the tubes. The cultures were photographed after 24 hours of growth, and the staining was photographed after drying overnight. In Figures 1, 3, 5, 7, and 9, the green arrow indicates how clarity increases with concentration among the experimental samples, and the red arrow indicates the same trend among the vehicle controls. This reflects a decrease in bacterial growth, likely due to the 95% ethanol. In Figures 2, 4, 6, 8, and 10, the tubes in the green box generally have stronger staining than those in the red box. This indicates that at 16 µL / mL, the experimental samples generally show more biofilm formation than the associated vehicle controls and suggest garlic extract's promotion of biofilm formation.

Trial 1

Figure 1: Trial 1 Cultures

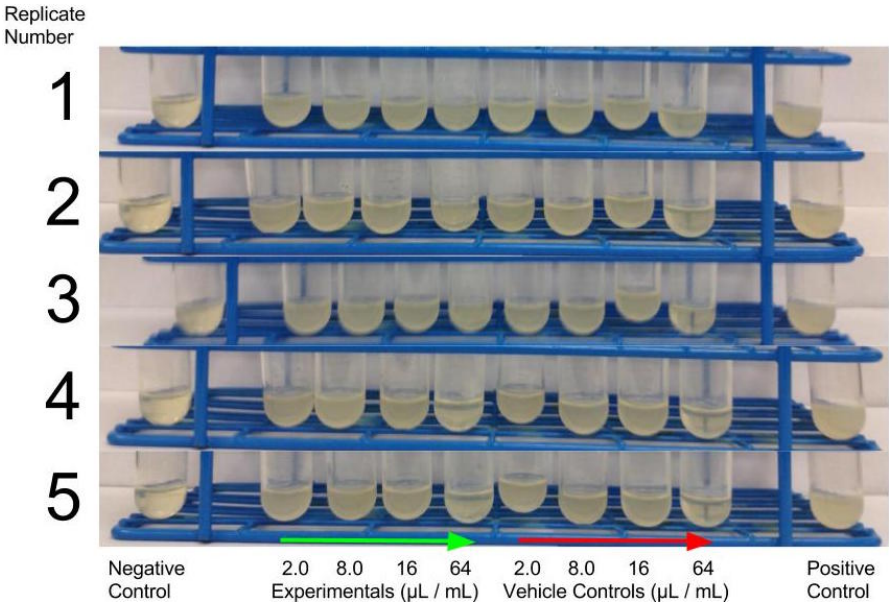
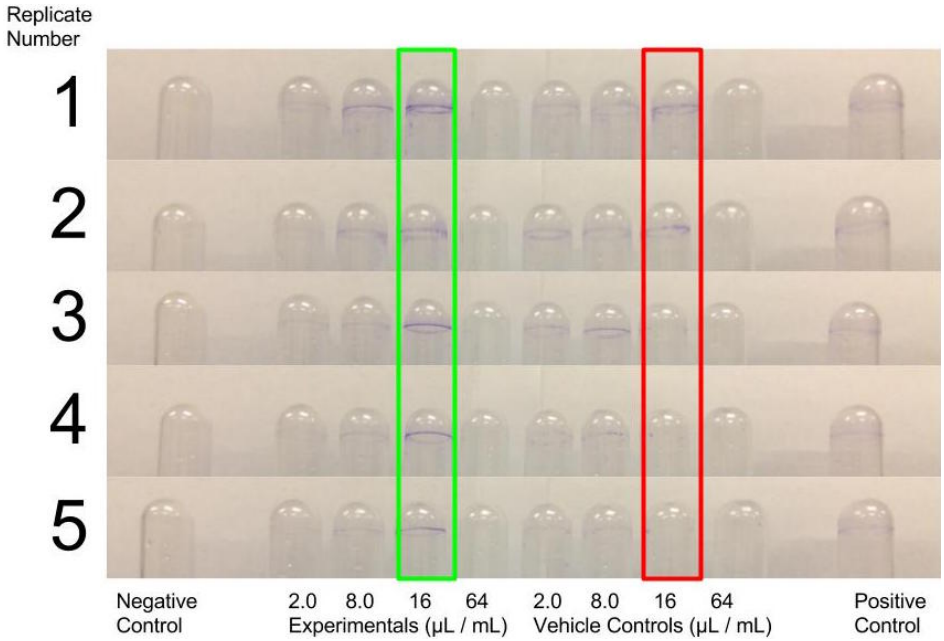


Figure 2: Trial 1 Staining



Trial 2

Figure 3: Trial 2 Cultures

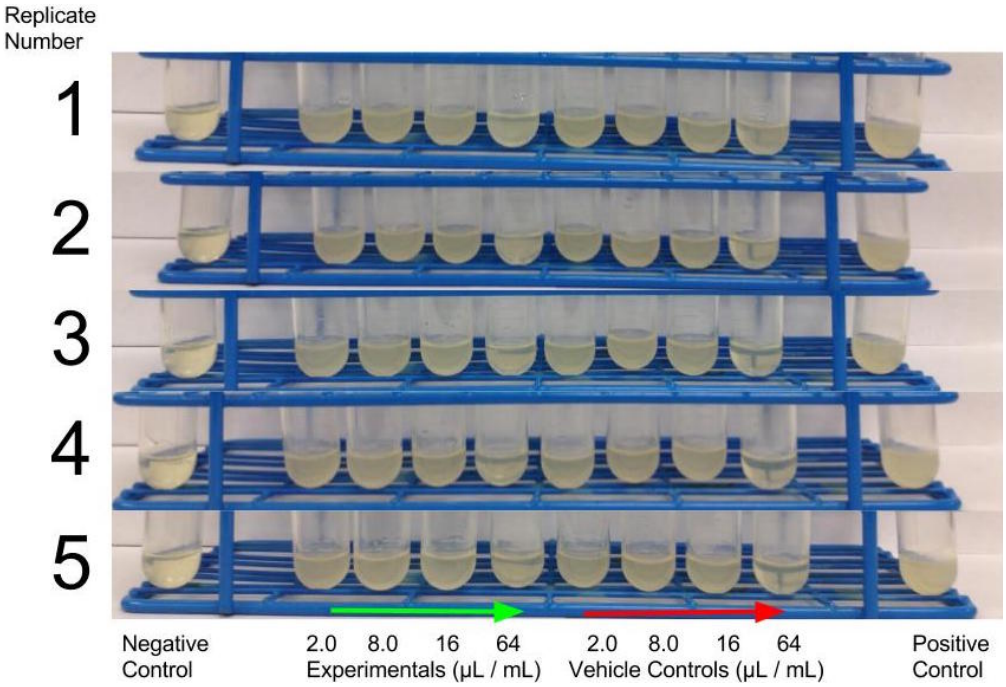
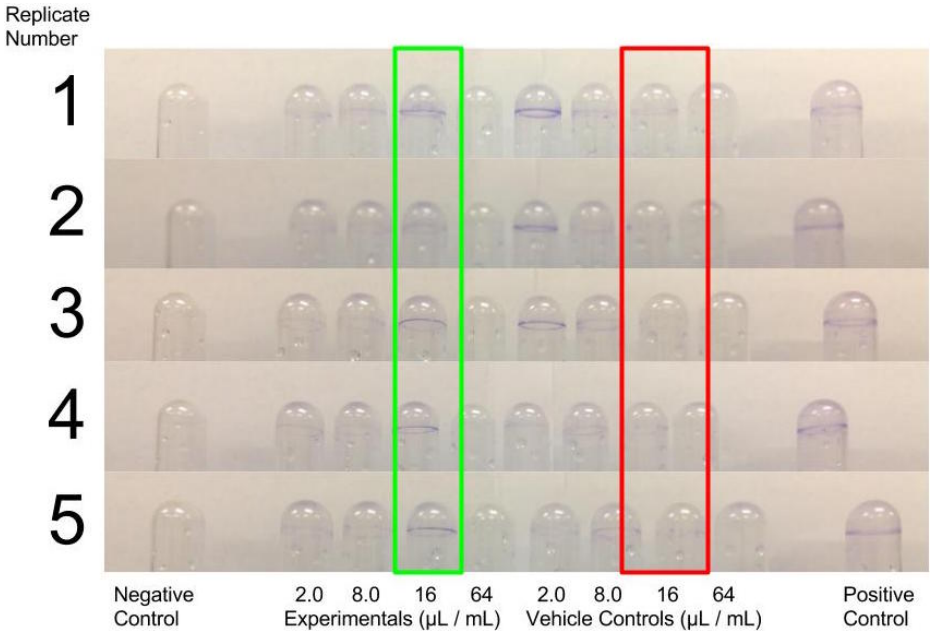


Figure 4: Trial 2 Staining



Trial 3

Figure 5: Trial 3 Cultures

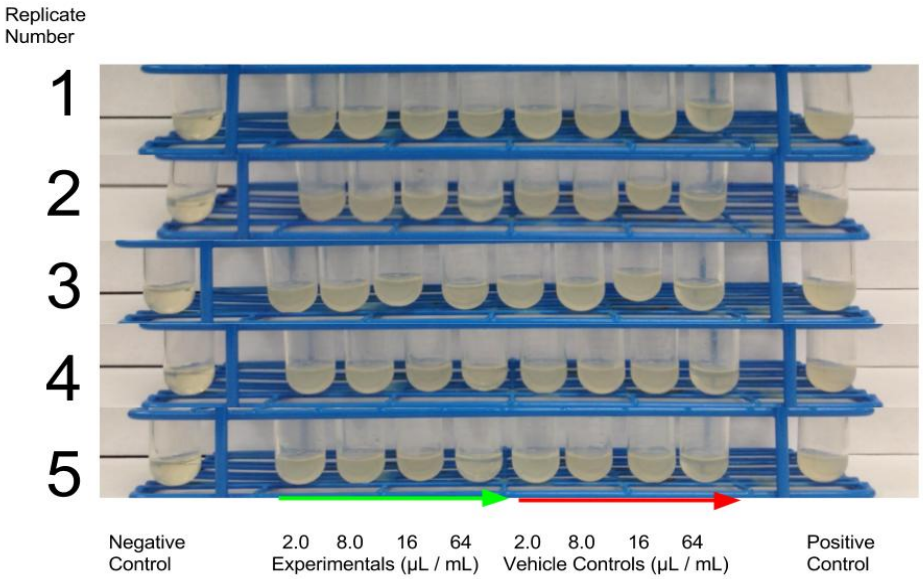
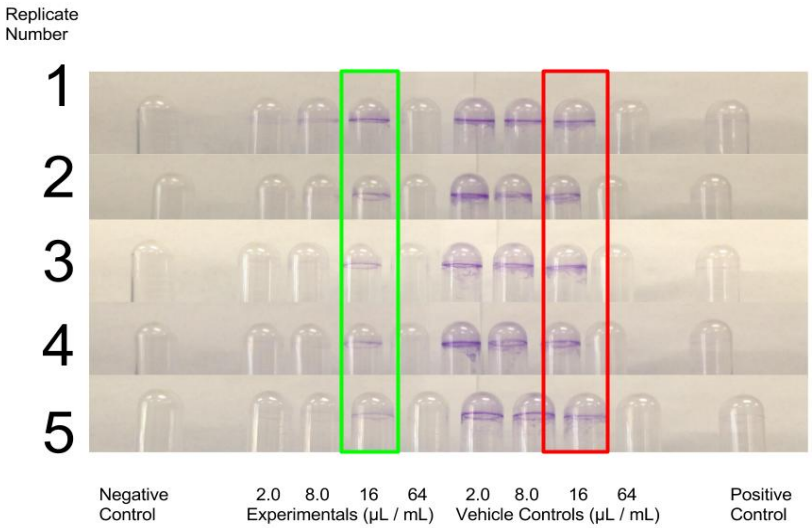


Figure 6: Trial 3 Staining



Trial 4

Figure 7: Trial 4 Cultures

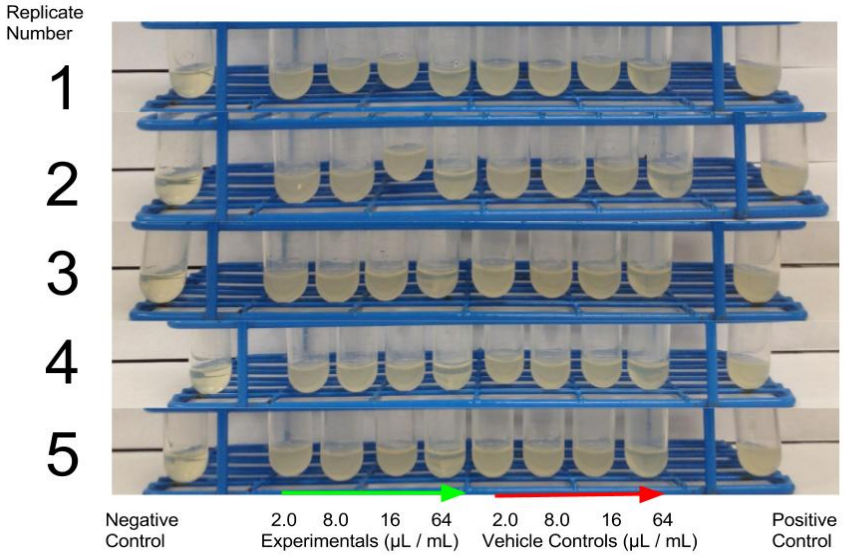
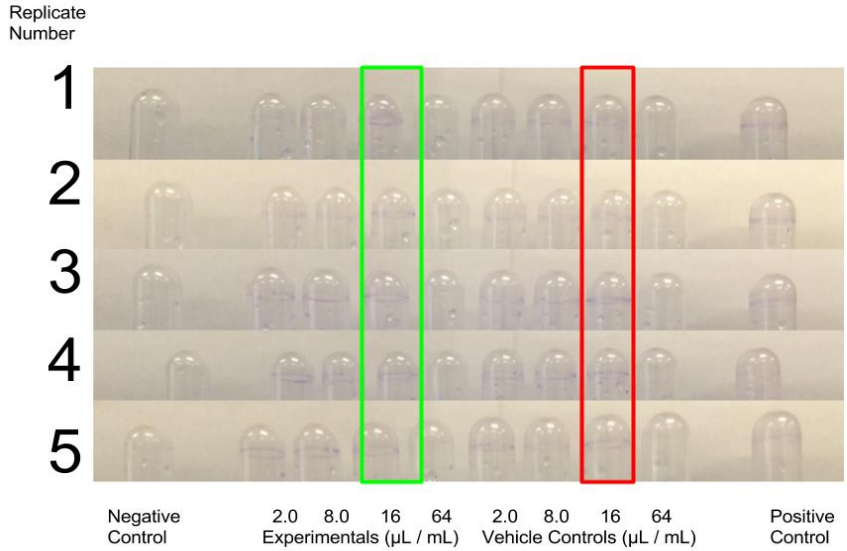


Figure 8: Trial 4 Staining



Trial 5

Figure 9: Trial 5 Cultures

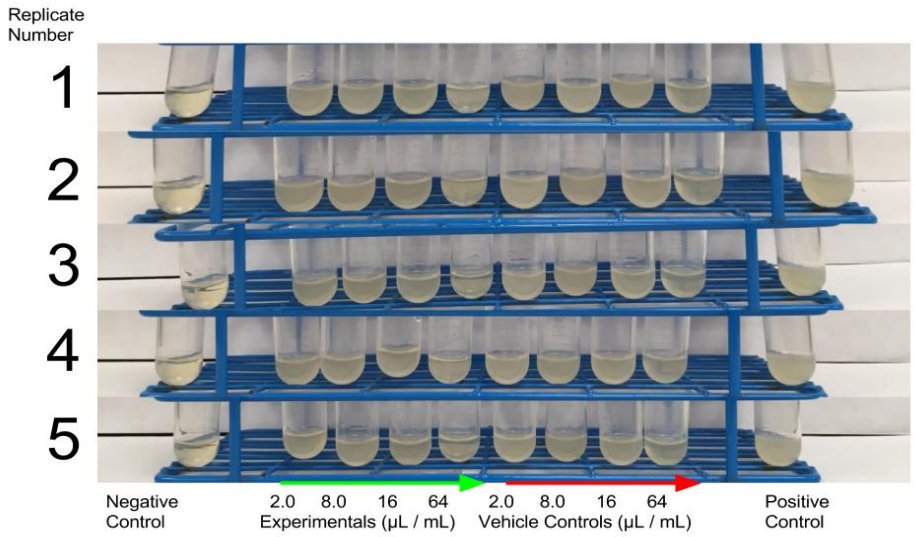


Figure 10: Trial 5 Staining