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Novel T4 bacteriophage and royal jelly infused disinfectant versus *E. coli* compared to QACs

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ABSTRACT

Antimicrobial resistance is an increasingly worsening phenomenon in the 21st century having resulted in thousands of deaths per year. Increases in the diffusion of antimicrobial resistance in gram-negative bacteria and prevalence of specific genes leading to resistance have been linked to an excessive usage of quaternary ammonium compounds (QACs) in gram-negative bacteria such as *Escherichia coli*. This experiment aims to develop a novel disinfectant solution (T4MRJP) that utilizes the entero-bacteriophage t4 and nine major royal jelly proteins (MRJPs) to inhibit the growth of *E. coli* on a MHA (Mueller Hinton Agar) growth medium. In the experiment, the Kirby Bauer Disk Diffusion Assay was first applied on six MHA plates inoculated with 36 evenly distributed susceptibility disks containing different concentrations of solution for three QAC groups, a positive t4 bacteriophage group, and two T4 groups at 90% and 98% dilution respectively. Results demonstrated a strong positive correlation between the increases of concentration to demonstrated inhibition but a lack of statistical significance between the T4 and QAC groups in regards to the proposed hypothesis. However, the diluted T4 group was just as effective as the QAC groups at inhibiting bacterial growth, especially at higher concentrations of the administered solution. In a second trial, however, the T4MRJP (Major royal jelly protein) cocktail was significantly less effective than the experimented QAC groups compared to the bacteriophage alone.

Introduction

Antimicrobial resistance is a natural phenomenon that occurs in various pathogens such as bacteria. This occurs because bacteria can evolve over time to gain resistance against antibiotics [1]. This increased resistance has been exacerbated in the 21st century due to an accelerated usage of antibiotic products in circumstances in which they are not always necessary. Their ubiquity has led to an overall spike in cases of antibiotic resistance and subsequently more deaths. In 2019 alone, antimicrobial resistance has been linked to over 5 million deaths globally because of excessive and unnecessary usage, thus causing an urgent public health threat [2]. Further, there was a 15% surge in U.S. infections caused by antimicrobial resistance during the first year of the COVID-19 pandemic [3], in which the increased use of antimicrobial disinfectants in healthcare settings was a factor [4].

Disinfectants, which include a diverse array of chemicals, such as quaternary ammonium compounds (QACs), have been increasingly attributed to a multitude of potential risks for antimicrobial resistance. For instance, the usage of QACs has been associated with increased antimicrobial resistance. This can be attributed to the presence of increased genes in bacteria that have aided in coding for potential resistance toward QACs, although the mere presence of these genes does not necessarily indicate that resistance will actually occur [5]. Nonetheless, the overall increase and identification of *qac* genes in various bacteria such as *Methicillin Resistant Staphylococcus aureus (MRSA)* and *Escherichia coli (E. coli)* suggest that this is a public health issue that should be tackled by potentially

considering alternatives [6][7]. Potential alternatives that may mitigate the risk of antimicrobial resistance have already been extensively researched. These include the utilization of bacteriophages and natural peptides like Royal Jelly, which have been shown to have antibacterial capabilities.

Bacteriophages and natural peptides have the potential to substantially reduce the amount of antimicrobial resistant bacteria in healthcare settings, due to their unique abilities to penetrate bacterial cell membranes in ways that antibiotics cannot [8][9]. Research findings in regards to these cocktail solutions could one day lead to the development of new disinfectants that will significantly reduce antibacterial resistance, and subsequently lower the number of deaths in healthcare settings throughout the world. The utilization of the entero-bacteriophage t4 (t4 bacteriophage), specifically, could provide a basis for potential usage against *E. coli* in healthcare settings.

This research study examines the ways in which a novel bacteriophage and Royal Jelly solution, named T4MRJP, interacts with *E. coli* bacteria. The study analyzes how the inhibition of *E. coli* via the T4MRJP solution differs as compared to only bacteriophages themselves and QACs.

Literature Review

Prevalence of qac-resistant genes

Qac resistant genes have become prevalent in both gram-negative and gram-positive bacteria. Further, research has demonstrated that *qac* genes can be found in combination with antibiotic resistance genes. In a review conducted by Cervinkova et al., for instance, *qac* genes were found to be identified in gram-negative bacteria like *Enterobacteriaceae* and *Pseudomonas Aeruginosa* that also had genes that code for resistance toward numerous antibiotics like β-lactams, a common antibiotic that inhibits cell wall development in affected bacteria [5]. This indicates that both antibiotic resistance and antiseptic (disinfectant) resistance often are linked together, thus demonstrating that both resistance types can be accelerated with the usage of *qac* disinfectants. Literature has also identified that the excessive usage of *qac* disinfectants can lead to a transfer of antibiotic resistant genes between bacteria. A study conducted by Han et al. in 2019 identifies that the implementation of five QACs promoted the bacterial conjugation transfer of the RP4 plasmid, a circular DNA strand found in bacteria that can code for genes for antibiotic resistance, thus demonstrating that QAC usage may help accelerate the occurrence of antibiotic resistance [10]. This is possibly because the usage of QACs led to a stimulation of the increased production of ROS (reactive oxygen species), reactive chemicals derived from O_2, which ultimately enhanced gene transfer between the bacteria[10][11]. This demonstrates that the increased usage of QACs can have influence in the increased diffusion of *qac genes.*

Both gram negative and gram positive bacteria have been identified to have *qac* genes. In a study conducted by Gahongayire et al., various bacterial species containing *qac* genes such as *Staphylococcus epidermidis* and *S. aureus* were identified on 6 conventional salon tools in Ishaka, Uganda [12]. In gram negative *Proteus mirabilis,* PCR assays identified 7 *qac* genes to be commonly present in the isolates ranging from 32.7 to 100% [12]. Overall, current literature identifies that the prevalence of *qac* genes is widespread and found in various commercial settings.
Research has also identified that the presence of *qac* genes are prevalent in numerous settings. In the case of *E. coli*, for instance, Zou et al. identified that four isolates of *qac* genes (*emrE*, *sugE(c)*, *mdfA* and *ydgE/ydgF)* were commonly found in 570 strains present on retail meats like chicken, pork, and ground beef found in the US in 2006 [7]. Similarly, in a study conducted by Ibrahim et al., isolates of *qac* genes (*qac ED1* and *qacA/B*) were identified in 70.6% and 14.7% respectively of the extracted *E.* coli strains of 1500 samples originating from diseased chickens diagnosed with colibacillosis, a type of avian flu caused by the bacteria [13].

The studies do not account for potential alternatives, but do highlight a growing problem. Given the prevalence of increased resistance in *E. coli* due to QAC usage, there is a need to develop another population management option.

Research surrounding bacteriophage usage

Bacteriophages are a commonly studied virus that specifically infect bacteria. Currently, phages are considered to be one of the most prevalent entities on Earth, as they occur in greater amounts at a factor of 10^{31} compared to bacteria and cumulatively infect bacteria at a rate of 10^{23} infections per second [14]. Phages have been seen to demonstrate great efficacy at inhibiting growth and potential ways to mitigate risks caused by antibiotic resistant bacteria [15]. In a case report conducted by Rodriguez et al, the utilization of a bacteriophage that specifically infected *MRSA* was able to effectively eliminate it one week after administration on an infected patient [16]. In another study by Ahiwale et al involving *P. Aeruginosa,* the utilization of the T7-like lytic phage (a phage that infects and takes over bacterial cells to replicate more of itself) was particularly effective in inhibiting the formation of any biofilms (cells sticking to a surface) containing the bacteria. Pre-formed biofilms were also completely destroyed [17].

For the inhibition of *E. coli,* numerous phages have been researched. The most commonly studied and extensively researched is the T4 bacteriophage. The t4 bacteriophage is a species of the Myoviridae family that has the potential to mitigate issues presented by antibiotic resistance in *E. coli.* Yap et al. identifies the t4 bacteriophage as a virus that specifically infects the *E. coli* bacterium via a penetrating motion. This occurs via a puncturing device constructed from a gp5 lysozyme bound to a Zn (zinc) atom, which initiates a drilling motion that allows for the destabilization of the phospholipid bilayer of the bacterium [8]. This destabilization ultimately enables the phage to destroy the *E. coli* bacterium with minimal resistance.

Research has shown that the utilization of the t4 bacteriophage on *E. coli* can have a multitude of benefits. In a study conducted by Dissanayake et al., a bacteriophage solution was able to decrease pathogenic viable *E. coli* counts in selected mice by up to 54%, which was a similar efficacy as ampicillin, a type of β-lactam antibiotic [18]. In another study conducted by Lisac et al., the introduction of the T4 bacteriophage resulted in a rapid decrease of the biofilm and cell concentrations to the point where detection was not possible [19]. This indicates that the utilization of the T4 bacteriophage serves as a potentially effective way to inhibit the growth of *E. coli,* thus serving as inspiration for this current experiment.

Antimicrobial capabilities of Royal Jelly, potential cocktail usage, and identification of a research gap

Royal Jelly (RJ) is a natural substance produced by the worker bees within *Apis Mellifera.* Although it is intended to be used for feeding larvae, literature has identified various antimicrobial capabilities against bacteria, especially in gram positive bacteria. This occurs because of the presence of 7 proteins in RJ , named Major royal Jelly Proteins (MRJPs), that have been identified to have antibacterial and antifungal properties. A study conducted by Brudzynski and Sjaarda identify, for instance, that the first encoded protein, MRJP1, was particularly effective at inhibiting the growth of *E. coli* and *Bacillus subtilis* because of contained antimicrobial peptides called Jelleines [20]. Another study conducted by Brudzynski et al. explored the inhibitory effects of honey glycoproteins within MRJPs that demonstrate antibacterial effects. Via a radial diffusion assay, which is used to determine antigen concentrations, they determined that honey glycoproteins harboring Jelleines were especially effective against gram negative bacteria that produced β-lactamase, an enzyme key for the development of antibiotic resistance in the membrane [21]. Additionally, a report conducted by Mureşan et al. demonstrates that jelleine peptides 1 and 2 inhibit various bacteria such as *E. coli, P. aeruginosa,* and *Klebsiella pneumoniae* at provided concentrations ranging from 2-30 µL/mL [22]. This demonstrates that the usage of such peptides could individually have antibacterial effects, as seen in **Table 1.** However, minimal research has been done to identify if their combination with another medium like a T4 bacteriophage could inhibit the growth of bacteria like *E. coli* more effectively.

Table 1. Identified Royal Jelly Protein, its molecular composition, and antimicrobial properties.

Royal Jelly Protein	*Composition*	*Antimicrobial Properties*	*Reference*
MRJP1	Jellein peptide (I,II,III) 3 N-glycosylated protein sites	Bacterial membrane permeabilization (ineffective against *E. coli)*	[22]
MRJP2	2 N-glycosylated proteins	Cell wall biosynthesis perturbation and aerobic respiration inhibition	[22]
MRJP3	RNA-binding domain	None	[22] [29]
MRJP4	8 N-glycosylated proteins and cysteine residues	Cell wall permeabilization on gram+ and gram-	[22] [30]
MRJP5	4 N-glycosylated sites	Cell wall permeabilization on gram+ and gram-	[22]
MRJP6	5 N-glycosylated sites	None	[22] [29]
MRJP7	3 N-glycosylated sites	Cell wall permeabilization on gram+ and gram-	[22] [29]

Research has been conducted to identify the efficacy of "cocktails" utilizing the T4 bacteriophage and other antibacterial mediums. For instance, a study conducted by Ryan et al. identified that a cocktail containing the T4 bacteriophage and the antibiotic cefotaxime was significantly more effective at reducing the growth of *E. coli* biofilms than just using the antibiotic itself [23]. Additionally, in another study conducted by Mangieri et al. found that forming a cocktail containing three *E .coli* targeting phages exponentially reduced pathogenic *E. coli* [24]. Another study utilized bacteriophages and chlorine, which also effectively reduced bacterial concentrations of *P. aeruginosa* [25].

As seen, although extensive research has been done to determine the efficacy of cocktails that inhibit bacterial growth using bacteriophages, antibiotics, or other mediums, there has been minimal research done that has utilized royal jelly and bacteriophages together to identify its relationship on inhibiting bacterial growth. Additionally, there is minimal research that identifies how the t4 bacteriophage compares with QACs in inhibiting bacterial growth, as current literature primarily only focuses on comparing QACs with other conventional disinfectants such as sodium hypochlorite (bleach) [22].

This study aims to bridge these gaps and analyze how the t4 bacteriophage itself firstly compares to QACs and how a cocktail of the t4 bacteriophage with royal jelly secondly compares to QACs in inhibiting the growth of *E. coli.*

Material and Methods

Trial 1 (T4 Bacteriophage and QAC comparison)

1. Solution Construction

6 groups of solutions were firstly constructed prior to inoculation of *E. coli* on the cultured agar plates. These included three QAC solutions extracted from their respective disinfectant,, a T4 bacteriophage control, and 2 experimental solutions, one being a T4 bacteriophage solution diluted to ensure a solution consisting of 90% water, and another being a second T4 bacteriophage solution that ensured 98% dilution. The control, which was a pure T4r coliphage solution purchased from Carolina Biological Supply, was not diluted. Lastly, three conventional disinfectants, as shown in **Table 2**, were selected based on the presence of their QACs, which were classified as being the only active ingredients in the solutions.

Table 2. Identified QACs in conventional disinfectants used in Trial 1 and their respective concentrations (AC=Ammonium Chloride)

Disinfectant	*QAC*	*Concentration*
Lysol (QAC 1)	Alkyl Dimethyl Benzyl AC	0.26%
Clorox (QAC 2)	n-Alkyl Dimethyl Benzyl AC	0.184%
	n-Alkyl Dimethyl Ethylbenzyl AC	0.184%
Shop and Shop (QAC 3)	Oxtyl decyl AC	0.0909%
	Dioctyl dimethyl AC	0.0364%
	Didecyl dimethyl AC	0.0545%
	Alkyl Dimethyl Benzyl AC	0.1212%

2. E. coli Culturing

The in vitro study first inoculated (applied) grown nonpathogenic K12 *E. coli* cultures on mueller-hinton agar (MHA). This would ensure individual safety and also ensure that the T4 bacteriophage would infect it. The cultures grown on six total plates would ultimately be used in the study to determine the overall results.

3. Assay methodology and Rationale

The Kirby Bauer Disk Diffusion Assay was used to evaluate and compare the efficacy of the T4 90 and 98% bacteriophage solutions to the QACs. This assay is intended to identify how much a certain medium inhibits bacterial growth. This is done by inoculating a paper disk with an antibacterial medium onto a cultured MHA plate, which would eventually produce a susceptibility zone (zone of inhibition). This is the region where bacterial growth does not occur because of the antibacterial effects. The larger the zone of inhibition is via radii measurements, the more inhibition

due to the medium occurs. Although this assay is primarily done to identify and compare inhibition levels of antibiotics, previous studies have utilized it to analyze the inhibitory effects of QACs. A notable example of this is done by Ramzi et al., in which this assay was used to specifically determine how much constructed QACs inhibited the growth of various bacteria. In the study, 10 µL of each solution were inoculated onto the disks via a micro pipetting method [26]. This served as a guide for utilizing the selected QACs in this experiment. Minimal research, however, has been done on the T4 bacteriophage's applications in the assay. 6 concentrations ranging from 10-30 µL each of both the 3 QAC and 2 T4 bacteriophage solutions were applied to sterilized paper disks (6 for each solution group) constructed from Whatman Qualitative Filter Paper that would later be used for inoculation on the *E. coli* grown on the MHA. The specific concentrations of the solutions inoculated were determined by an interval of four, going from 10 µL/mL, 14 µ/mL, 18 µL/mL, 22 µL/mL, 26 µL/mL, to 30 µL/mL. This ensured that inhibition could occur, as noted by Mureşan et al. [22]. Inoculation of both variables were intended to ensure that contamination did not occur through swabbing. A marker was then used to identify the specific concentrations used for each of the inoculated solutions. The cultures were then incubated at a temperature of 37℃ over 18 hours to determine if an identifiable zone of inhibition would subsequently be analyzed.

4. Control population

A separate plate cultured with *E. coli* not receiving either the QAC or constructed diluted bacteriophage solution was used to act as a control to address the null hypothesis. Similarly, six concentrations of the purchased pure bacteriophage solution with the same volume ranges were inoculated on 6 disks applied to an MHA plate. This plate was also incubated at 37℃ over 18 hours.

5. Measurement of inhibition

The zone of inhibition was measured in mm for each of the six inoculated plates of the three QAC solutions, 2 T4 solutions, and the control after incubation. Utilization of rulers allowed for susceptibility and resistant analysis of the remaining cultures. The radii were recorded on a datasheet and allowed for analysis of the efficacy in regards to the extent each solution inhibited growth of the cells.

6. Statistical Analyses

To identify statistical significance and correlation ($p<0.05$ for statistical significance) to determine if efficacy was warranted, a one-way analysis of variance (ANOVA) was performed to identify the influence the application of the 2 T4 bacteriophage solutions had on overall bacterial inhibition when compared to both the 3 QAC solutions and control. This was conducted because of the presence of three groups used in the experiment. A student's t-test was also used to compare each independent variable/group to each other to determine if the inhibition between the two did not simply occur by chance.

7-Disposal

The remaining bacterial cells receiving the T4-MRJP-1 solution and QAC were all autoclaved to ensure sterilization. The controlled *E. coli* group and remaining solutions were further autoclaved. Each of the agar plates were subsequently disinfected with a bleach solution to ensure sterilization of the surfaces.

Trial 2 (T4MRJP cocktail and QAC comparison)

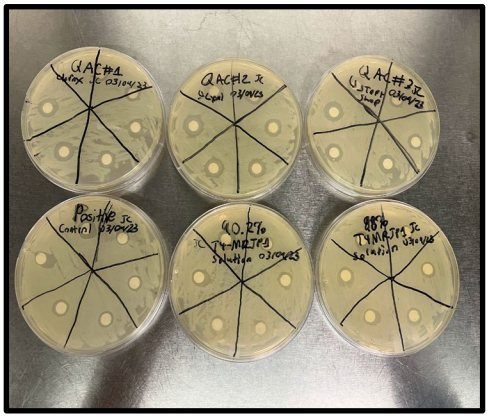
A second trial utilizing the same methodology outlined in steps 2-7 was used to test the experimental cocktail solution utilizing the t4 bacteriophage and royal jelly (named T4MRJP). This solution, similar to the individual experimental

T4 bacteriophage solution used in Trial 1, was also diluted to 90% and 98% respectively. Moreover, the bacteriophage and royal jelly were composed of equal concentration for the remaining 10% and 2% respectively of the parts not diluted.

Results

The aim of this experiment was to determine whether the solutions utilizing MRJPs and the t4 bacteriophages were more effective at inhibiting *E. coli* than conventional AC based disinfectant solutions and to see how well the solutions minimized further bacterial growth. Trial 1 was conducted at the BioNtech biotechnology laboratory in Groton, Connecticut. All instruments used for the experiment were further disinfected via an autoclave or 70% isopropyl alcohol.

For trial 1, all 5 of the tested experimental disks and singular control MHA dishes cultured with *E. coli* and the radii of the zones of inhibition were recorded (**Table 3**). In each of the groups, the maximum radius was less than 7mm, indicating that the susceptibility ranges were lower than the ranges produced by disks containing a greater average concentration of antimicrobial agents (100 uL). There was a strong positive correlation regarding the increase in the diameters of the zones of inhibition with an increase in the amount (in µL) administered (R^2=0.860) derived from the average Fisher's *z*=1.297). This correlation in the increase was greatest in the 90% diluted T4 solution (r=0.971) and lowest in QAC 1 (Dimethyl Benzyl AC and Ethylbenzyl AC) (R^2=0.575). Pictures of the inoculated disks were also taken and provided in **Figure 1** following incubation at 37℃ for 18 hours at 95% humidity.



Trial 1

Figure 1. Inoculated disks of *E. coli* and susceptibility zones following incubation at 37℃ for 18 hours at 95% humidity for Trial 1. Configuration is as follows:

Top-left disk- QAC 1
Top Middle Disk- QAC 2
Top Right Disk- QAC 3
Bottom left disk- Positive Control (pure T4 bacteriophage)
Bottom middle disk-T4 Bacteriophage solution (90% water concentration)
Bottom right disk- T4 Bacteriophage solution (98% water concentration)

*Radii recordings at 10 uL-30 uL for each solution started from the first quadrant and were recorded clockwise for Trial 1.

Table 3. Identified measurement of radii (mm) for zones of inhibition of administered solutions at various volumes in Trial 1. An interval of an increase in 4 uL was used starting from 10 uL-30 uL was used to determine the radii for the five experimental groups and one positive control group.

Solution Type	10 µL	14 µL	18 µL	22 µL	26 µL	30 µL
QAC1: Clorox:Dimethyl Benzyl AC+ Ethylbenzyl AC (0.368%)	2	4	4	3	4	4
QAC2: Lysol: n-alkyl dimethyl benzyl AC (0.26%)	2	3	3	4	4	4
QAC 3: Stop&Shop: Octyl decyl dimethyl AC+ Dimethyl Benzyl AC (0.212%)	1	2	3	3	4	3
Positive Control (acquired T4 bacteriophage)	2	2	1	2	6	4
T4 solution (90% water concentration)	1	2	2	3	4	4
T4 solution (98% water concentration)	1	2	2	4	3	6

The ranges and means were also calculated based on the acquired data in the first trial. These values are displayed in **Table 4.**

Table 4. Mean radii and range of susceptibility zones in trial 1 (mm). As shown, the QAC experimental group is denoted as A1, the positive control as X, and the T4 group as B1.

Trial 2 (T4MRJP Cocktail)	A1	X	B1
Mean Radii of Susceptibility Zones (mm)	3.167	2.833	2/833
Range of susceptibility zones (mm)	1-4	2-4	1-6

The mean radii of the zones of inhibition were the same in X and B1 at 2.833 mm, but lower than A1 at 3.167 mm. The range in B1, moreover, was greater than in both X and A1, thus indicating a greater variety in the zones of inhibition than in the X and A1 cocktail. Furthermore, although a true MIC (Minimum Inhibitory Concentration) cannot be determined due to limitations of the Kirby Bauer Disk Diffusion Assay, an estimated MIC can be calculated based on the the susceptibility zone that produced the least inhibition given a certain concentration of a solution. In trial 1, the greatest prevalence of the MIC was determined to be at 1 mm, which occurred the most given 10 uL of QAC 3 and both T4 solutions. To determine significance ($p<0.05$) between the identified efficacy of t4-MRJP in inhibiting the growth of *E. coli* in comparison to the experimental QAC solutions and control, a one-way analysis of

variance (ANOVA) was performed to determine the F-Value and whether the null hypothesis should be rejected. The null hypothesis was that the proposed T4 solution would not produce a greater zone of inhibition. Using the calculated MS_B and MS_w values, the F ratio was calculated via the formula $\frac{MSb}{MSw}$. The resulting F ratio was 0.300 with a related p-value of 0.743 (>0.05). This indicated that the null hypothesis failed to be rejected and that there was no significant correlation between the groups in its inhibitory effect. Further, using a student's t test, the statistical significance of each T4 solution was also calculated in relation to only the control group and each QAC solution respectively instead of each independent variable (IV) combined like in the ANOVA test **(Table 5a).**

Table 5a. Identified p-values given the two compared independent groups (<0.05 indicates significance) for Trial 1.

Compared IVs (Trial 1)	p-value determined from student's t-test
T4 solution (90%) + Control bacteriophage solution	0.856
T4 solution (98%) + Control bacteriophage solution	0.877
T4 solution (90%)+QAC 1	0.196
T4 solution (90%)+QAC 2	0.290
T4 solution (90%)+QAC 3	1.000
T4 solution (98%)+QAC 1	0.549
T4 solution (98%)+QAC 2	0.687
T4 solution (98%)+QAC 3	0.701

Table 5b. Identified p-values given the two compared independent groups (<0.05 indicates significance) for Trial 2.

Compared IVs (Trial 2)	p-value determined from student's t-test
T4MRJP (90%)+Control bacteriophage solution	0.602
T4MRJP (98%)+Control bacteriophage solution	0.762
T4MRJP (90%)+QAC 1	0.194
T4MRJP (90%)+QAC 2	0.853
T4MRJP (90%)+QAC 3	0.764
T4MRJP (98%)+QAC 1	0.443
T4MRJP (98%)+QAC 2	0.884
T4MRJP (98%)+QAC 3	0.245

As seen by table 5a and 5b, for all determined p-values calculated by student's t-test, the results remained statistically insignificant, thus demonstrating that neither the T4 solution or T4MRJP solution was more effective than either the control or conventional QACs at inhibiting *E. coli* growth for all tested concentrations.

In trial 2, results were acquired via the Kirby Bauer Disk Diffusion Assay for the tested T4MRJP solution and the QACs at a constant 25 µL/mL. This occurred because of structural limitations. This data was similar to that recorded in trial 1. The radii of the zones of inhibition were recorded in all 36 disks tested in trial 2, as seen in **Figure 2.** A picture of each of the 6 experimental MHA plates is also provided in **Figure 3.**

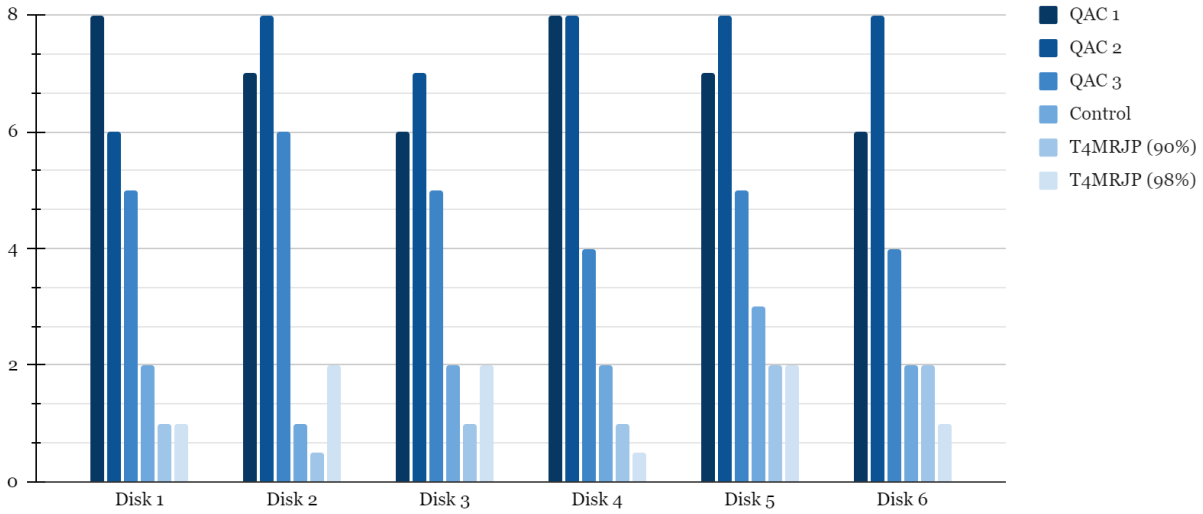
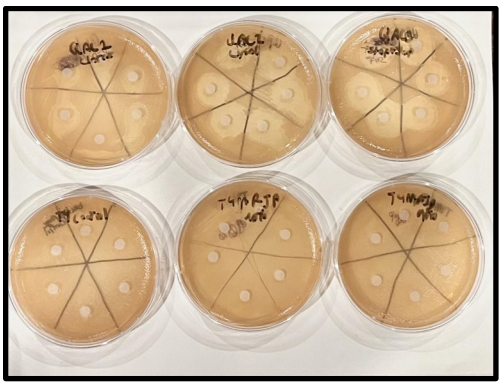


Figure 2. Identified radii of zones of inhibition (in mm) for tested experimental groups at given 25 µL/mL for each disk on respective agar plate.



Trial 2

Figure 3. Inoculated disks of *E. coli* and susceptibility zones following incubation at 37℃ for 18 hours at 95% humidity for Trial 2. Configuration is as follows:

1. Top-left disk- QAC 1
2. Top Middle Disk- QAC 2
3. Top Right Disk- QAC 3
4. Bottom left disk- Positive Control (pure T4 bacteriophage)
5. Bottom middle disk- T4MRJP solution (90% water concentration)
6. Bottom right disk- T4MRJP solution (98% water concentration)

Similarly to Trial 1, the mean radii was identified for each of the three solutions as noted in **table 6.**

Table 6. Mean radii for A1, X, and B1

Trial 2 (T4MRJP Cocktail)	A1	X	B1
Mean Radii of Susceptibility Zones (mm)	3.875	2.3	0.83

For statistical analysis, the one way ANOVA comparing the 2 T4MRJP groups to the 3 QAC groups and 1 Control group determined that the specific p-value was not statistically significant (p=0.874). Additionally, the student's t-test determined that the comparison of the variables was not statistically significant as well, as seen in **table 5b**. This means that it is likely the results occurred by chance and was not a direct correlation. As a result, the alternative hypothesis stating that the novel T4MRJP solution would be more effective in terms of inhibiting *E. coli* growth was rejected. However, similar in Trial 1, this does not indicate that the solution is not effective as inhibition was still reached. Due to the concentration applied to each disk remaining constant at 20 µL/mL and the lack of an alternative microdilution assay, an MIC could not be estimated.

Discussion

The results from trial 1 demonstrate that the T4 solution was primarily less effective than the tested QACs at inhibiting the growth of *E. coli* as indicated by the differentiation in the mean radii between B1 (3.167 mm) and A1 (2.833) as seen in **Table 4.** The T4 solution, at the 98% dilution level, did inhibit a greater amount of bacterial growth at 30 microliters administered than that of the QACs tested, thus indicating that this solution could be utilized at a higher concentration as an alternative of conventional QACs. This is evident in **Table 1,** where the identified radius of the susceptibility zone for the 98% T4MRJP solution was 6 mm at 30, 2 mm larger than QAC 1 and 2, and 3 mm larger than QAC 3.

The inhibitory effects of the 98% concentrated T4 solution are comparable to a spray solution containing 0.4% QAC, as the measured diameter was 9.0 mm tested in Ramzi et al. It should be noted, though, that the tested disinfectants produced smaller zones of inhibition than Ramzi et al, but were not denoted as ineffective, as indicated by the presence of a R (resistance level) value. This indicates that the specifically constructed T4 solution concentrated at 98% is comparable in terms of efficacy via the inhibition of *E. coli* to conventional QAC disinfectants tested in the experiment and in current literature such as in Ramzi et al [26]. However, as determined by statistical analyses tests and the one-way ANOVA ($p>0.05$), the T4 solution is not more effective than any of the identified QACs as predicted by the alternative hypothesis.

Additionally, the results of the study, specifically in Trial 2, demonstrated that the inclusion of the royal jelly had a negligible, even negative, effect on the efficacy of inhibiting bacterial growth. This is indicated by a calculated $p>0.05$ for the T4MRJP (90%) and T4MRJP (98%) solutions compared to the positive control group, which deems that the inclusion of the bee protein had an insignificant impact on increasing the efficacy of the t4 bacteriophage solution. Additionally, observation of the mean values in **Table 6** suggested that B1 (the experimental T4MRJP group) was less effective than the experimental T4 group in Trial 1 as the average radius of the inhibition zone was less ($0.83<2.833$). The only potentially plausible evidence for increased efficacy of T4MRJP in inhibiting *E. coli* compared to the positive control would be the greater range of the susceptibility zones in B1 (QAC groups) than X (control). However, it is very unlikely that this is a definite reason and did not just occur by chance due to factors such as the temperature of the incubator (37 degrees) and its humidity levels.

Limitations

The conducted experiment is limited in that the novel solution was only tested against *E. coli* bacteria due to a lack of feasibility. Various research studies testing antibacterial disinfectants test them on multiple mediums of primarily similar bacteria to *E. coli* such as other gram- bacteria like *Pseudomonas aeruginosa,* which was included in studies such as Ahiwale et al and Zhang et al [17][25]. This indicates that limited information on the efficacy of T4MRJP in inhibiting the growth of other bacteria is unknown, as the study was specifically constructed to address *E. coli* bacteria. Additionally, limited groups of QACs serve as a limitation for the results of the experiment. Although reproducible, only 3 QACs were addressed, which limited the scope of the experimentation.

Additionally, the study is tailored to only identify the results of inhibition of *E. coli* via the T4 bacteriophage. The results are limited in that only the T4 bacteriophage was used. This is simply because of the required host specificity of the bacteriophage. As indicated by Loc-Carrillo & Abedon, the narrow parameters for host infection of the bacteriophage can make it very difficult to be utilized conventionally. Nonetheless, this study provides insight on the development and efficacy of a singular type of bacteriophage and its potential usage [28] .

There are other limitations regarding the determined MIC value. As previously stated, the identified MIC was merely an estimate due to incapabilities of properly calculating it via the Kirby Bauer Disk Diffusion Assay. To properly determine an accurate MIC, the broth microdilution methodology would have also had to be implemented in the study, as noted in Rotilie et al [27]. Construction of homemade disks via Whatman qualitative filter paper additionally could have produced limitations. This was done because there was a lack of availability of them commercially.

Future Directions

Although the addition of royal jelly to the T4 solution was ineffective in increasing its inhibitory ability against *E. coli*, the findings provide new research on the potential development of bacteriophage cocktails that could be more effective in inhibiting bacterial growth. Based on the results of research such as Mangieri et al. and Ryan et al., the development of new cocktails utilizing the bacteriophage are sorely needed and promising for improving health sanitation outcomes. This study prompts and identifies an answer to the question of whether royal jelly could actually

increase the extent of inhibition. Even though significance was not reached, future research can continue to explore potential cocktail solutions involving bacteriophages. Lastly, more research can be done in the future to analyze how the T4 bacteriophage specifically compares in efficacy to other QAC-based disinfectant solutions that were not used in this study.

Conclusion

Although the results of the conducted experiment suggest that the novel T4MRJP solution is not more effective than conventional QAC disinfectants, they do demonstrate that t4 bacteriophages can be utilized as an effective way to inhibit the growth of *E. coli*. Additionally, utilizing the MRJP protein, although not significant in this study, can potentially be significant in future studies not involving *E. coli.* Furthermore, given the age of increasing antimicrobial resistance due to the excessive use of disinfectants like QACs, the potential introduction of more novel solutions and potentially successful ones other than T4MRJP can be developed. Additionally, the efficacy of the T4 bacteriophage can be substantiated as in both trials, since it produced a similar zone of inhibition as the QAC solutions. This further suggests that the implementation of the T4 bacteriophage could be potentially utilized effectively in healthcare settings in upcoming years to improve outcomes for those most at risk.

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