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Testing the effectiveness of antimicrobial agents against common microorganisms

Paige Schotanus and Aunika Venables

This study evaluated the efficacy of antimicrobial agents against common lab and oral microbes. Samples of oral bacteria were collected and streaked on agar to obtain single colonies used to establish pure liquid cultures. Antibiotic discs were placed on agar lawns of pure cultures and incubated before measuring the zones of inhibition. Subsequent studies examined filter paper discs absorbed with 25-100% ethanol tested on agar lawns of the same pure oral cultures. This methodology was repeated with ethanol discs on lawns formed from *Bacillus subtilis*, *Escherichia coli*, and *Micrococcus luteus*.

Disinfection is essential to decrease the transmission of pathogenic microbes and promote sterility. It is common to use antibiotics to treat bacterial infections and other antimicrobial agents, such as ethanol, to disinfect workspaces. A concentration of 70-80% ethanol has been demonstrated as the most effective[1]. Alcohols act as antimicrobial agents by inducing cell membrane damage, denaturing proteins, and causing other protein-alcohol interactions[2].

This study aimed to examine the effective concentrations of ethanol against common microorganisms and to identify oral bacteria samples as Gram-positive or Gram-negative. To achieve this, a mix of novel procedures, including oral bacteria isolation, and established protocols, such as Gram staining, were used.

Sample Collection

All procedures were completed using sterile equipment and aseptic laboratory practices. Samples of oral bacteria were collected from two female participants of similar age and ethnicity. A cotton swab was swiped three times over both tonsils and the back of the throat (Fig. 1A). A separate cheek sample was taken by swiping both cheeks three times. The two swabs were placed in individual conical vials containing 5mL of Difco Nutrient Broth (Fisher Scientific) and inverted five times to mix. The four samples were incubated in a water bath at 37°C for 24 hours. These conditions were used for all liquid oral bacteria incubation.

Preliminary Methodology

After incubation, the oral bacteria were mixed by inversion five times. Each sample was streaked to isolate single colonies on Difco Nutrient Agar (Fisher Scientific) following the protocol outlined by Wessner et al. (2016) (Fig. 1A)[3]. The plates were incubated, agar on top, for 24 hours at 37°C. These conditions, and agar type, were used for all oral bacteria plates.

Post incubation, pure liquid cultures were established by individually removing three single colonies from each streaked plate to inoculate 5mL of broth as outlined by Leboffe and Pierce (2016) (Fig. 1A)[4]. After incubating, two lawns of each cheek and throat pure culture from both participants were created by adding 200μL of pure culture to the surface of agar plates then distributing evenly using a Bacti Cell Spreader (Fig. 1A). Two antibiotic discs, 30μg of vancomycin and 10μg of gentamicin (Becton
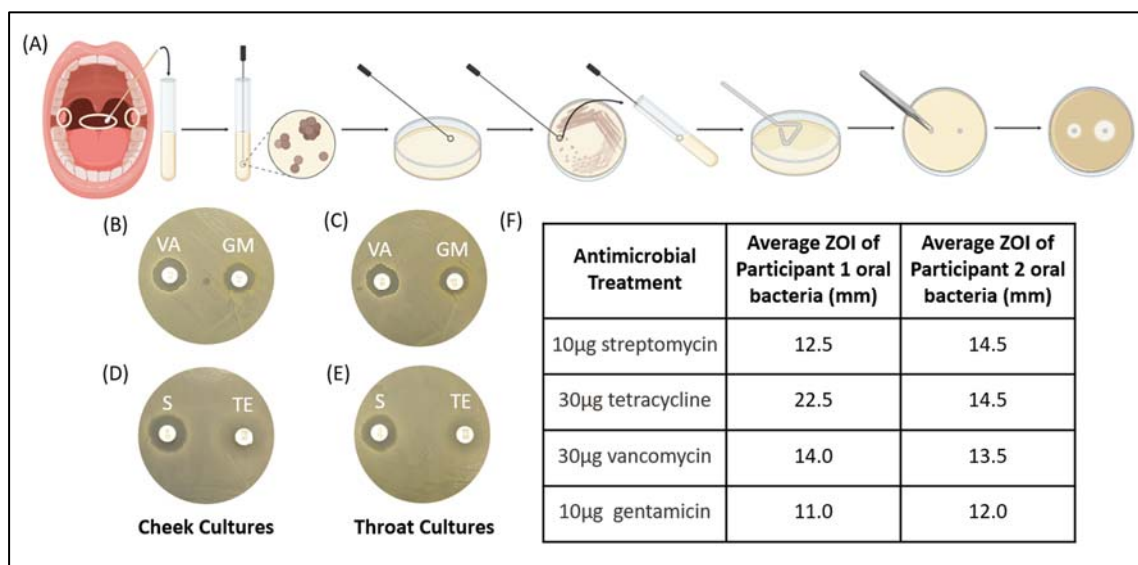
Fig. 1 | Preliminary methods and antibiotic ZOI results. (A) Samples of cheek and throat bacteria (throat sampling locations circled in white) were collected and streaked on agar to obtain single colonies to make pure liquid cultures. Antibiotic discs were placed on lawns of pure cultures and incubated before measuring the ZOIs. (B)Throat and cheek ZOIs formed by vancomycin (VA), gentamycin (GM), streptomycin (S), and tetracycline (TE), as labeled. All ZOI plate images are from Participant 2. (F) Table showing the average antibiotic ZOI, in millimetres, for cheek and throat cultures for each participant.

Dickinson), were placed on one lawn of each cheek and throat sample. The remaining lawns had one 30µg tetracycline disc and one 10µg streptomycin disc. The lawns were incubated, agar on bottom, before measuring the diameter of cleared bacteria around the discs, called the zone of inhibition (ZOI) (Fig. 1B-E).

Staining Procedure and Oral Bacteria Morphology

Slides of individual colonies from streak plates were prepared and stained using a standard Gram-staining protocol with crystal violet, iodine, 95% ethanol, and safranin followed by microscopic examination.

Both participants had two distinct bacterial types isolated from each cheek and throat agar plate. It is believed these two species are the same across participants as response to most applied antimicrobial agents and staining observations were consistent for each sample. Additional bacterial species could have been present in initial samples but

did not survive as a result of the conditions cultures were subjected to.

Of the two distinct colony types, one presented macroscopically as larger, lighter tan-coloured colonies and the other as smaller, darker tan-coloured colonies. The larger bacteria were generally found in dense clumps with coccus morphology. The smaller bacteria were typically found in sparse clumps with cocci morphology but were generally more ovoid. The two types of bacteria observed were both identified as Gram-positive. Based upon these observations, it is hypothesized the larger were *Staphylococcus sp.* and the smaller colonies were *Streptococcus sp.*.

Antibiotic ZOI Results

All antibiotics produced a ZOI on participant oral bacteria lawns; both cheek and throat cultures produced similar responses to the different antibiotics (Fig. 1F). Further, between participants, all results were similar except for tetracycline which had
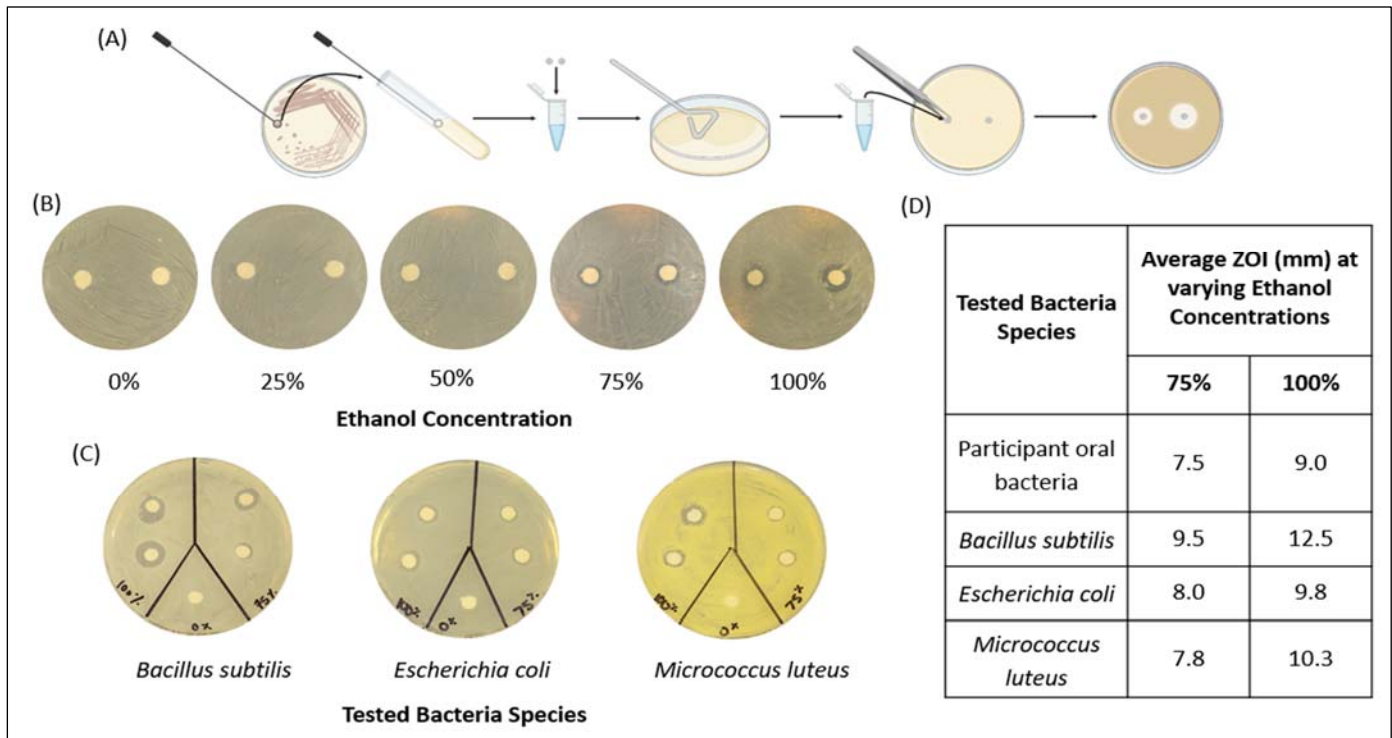
Fig. 2 |Ethanol Testing Methodology and Results. (A) Initial bacteria streaks were used to create pure liquid cultures. Discs were soaked in different concentrations of ethanol and agar lawns were made from pure cultures. The ethanol discs were placed on the lawns, incubated, and ZOI were measured. (B) The ZOI of Participant 1's results of differing ethanol concentration on throat bacteria for the primary ethanol testing. ZOI were only observed at the 75% and 100% ethanol concentrations for both participant oral bacteria. Between participants, results were similar for each concentration. (C) The ZOI formed by 0% (middle), 75% (right), and 100% (left) ethanol on lawns *of B. subtilis*, *E. coli*, and *M. luteus* of the secondary ethanol testing. ZOIs formed for 75% and 100% ethanol discs for all types of bacteria. *E. coli* and *M. luteus* had circular shaped ZOIs. The ZOIs for *B. subtilis* were not circular and slightly irregular. (D) Table showing the average ZOI (mm) of the tested bacteria species at different concentration of ethanol. The throat culture bacteria were on average the most resistant and the greatest effect was seen for *B. subtilis* at both concentrations.

a greater difference of ZOI in comparison to the other antibiotics. This difference between participants and small sample size suggests a larger sample size is needed to verify these results. The variability in bacterial responses to tetracycline could be unrelated to the species of bacteria present but rather affected by physiological differences between participants.

The streaked cheek swab cultures grew less compared to the throat samples for both participants. Since more robust bacterial growth was desirable for the purposes of this study, only throat cultures were used for the remaining methodology involving ethanol.

Ethanol Testing Methodology

Another set of pure throat cultures from each participant were formed and incubated in the same manner as previously described in the preliminary methodology using colonies from the original streaked agar plates. After incubation, five lawns for each participant were created as described above. Four 6mm filter paper discs were soaked for 30 minutes in separate 1.5mL Eppendorf tubes each containing separate 1mL solutions of 25, 50, 75, and 100% ethanol, with water was the control. The soaked ethanol discs were removed from the tubes using forceps, and two of each concentration were

immediately placed on the same lawn for each participant totalling ten lawns with two ethanol discs each. The lawns were incubated for 24 hours at 37°C followed by ZOI determination. (Fig. 2A).

Ethanol Testing Results

Results determined only ethanol concentrations of 75% and 100% created a ZOI while 25% and 50% were unchanged compared to control (0%) (Fig. 2B).

A third set of experiments using the procedures outlined for Ethanol Testing were completed on bacterial lawns formed from wild-type strains of *B. subtilis*, *E. coli*, and *M. luteus.* The lawns were created each with a standardized bacterial concentration between 0.08-0.10 AU at 610nm measured with a spectrometer.

B. subtilis, a Gram-positive spore-forming bacteria, did not perform similarly to the other Gram-positive cultures (participant bacteria and *M. Luteus*) (Fig. 2C). Based upon ethanol's mechanism of action and the lack of an outer membrane, *B. subtilis* was expected to have a smaller ZOI compared to Gram-negative bacteria[5,6]. Additionally, *B. subtilis*, the only spore-forming bacteria tested, did not result in greater resistance as demonstrated in other studies[7]. Further studies with *B. subtilis* are necessary to confirm this unexpected finding.

The participant throat bacteria had a smaller average ZOI for the tested ethanol concentrations suggesting it was more resistant than the three strains of laboratory bacteria (Fig. 2D). This could be a result of lab strains losing virulence genes that are typically carried by bacteria, while fresh participant bacteria may be better equipped to resist the ethanol treatments[8].

Conclusion

Through the development of a preliminary methodology and subsequent experiments, this study supported ethanol concentrations of 75% and above

as appropriate antimicrobial agents. Ethanol was able to kill the oral bacteria of participants demonstrating the effectiveness of products like alcohol-based hand-sanitizers. This study further shows ethanol has broad spectrum antimicrobial capabilities[9], specifically against Gram-positive, Gram-negative, and spore-forming bacteria.

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An in vitro investigation of the antibacterial efficacy of proanthocyanidins in raspberry extracts against *Escherichia coli*

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Current urinary tract infection (UTI) diagnostics involve urine sample incubation and subsequent analyses for *Escherichia coli* presence. While awaiting confirmation of analyses, patients often resort to home remedies (e.g. raspberries) which contain proanthocyanidins (PACs), the active antibacterial agent. In this study, UTIs in human urine were analogued by spiking urine samples with *E. coli* at an experimentally determined optimal ratio of 90:10. The PAC localization and relative quantification of efficacy for different raspberry extracts (i.e., juice, liquid pulp/pomace, seed) was qualitatively evaluated using a novel methodology, which combined agar streaking, spectrophotometric quantification of absorbance readings, and high-throughput plate reading. Raspberry juice was found to exhibit the highest antibacterial activity, with a direct correlation between concentration and inhibition of *E. coli* growth.

Context

90% of UTIs are caused by proliferative growth of Escherichia coli within the urinary tract.[1] Patients exhibit characteristic symptoms: burning sensation, frequent urination, and cloudy, dark urine. Urinalysis is the recommended first-tier diagnostic method; however, results can take between one to three business days. Patients seeking quick symptom alleviation from the intense discomfort from presence of *E. coli* within the lower urinary tract often resort to at-home remedies.[1] Popular remedies involve cranberry juices or capsules. By investigating the antibacterial properties of other PAC-containing fruits, such as raspberries, further at-home remedies can be explored to treat UTI-associated symptoms.[2]

Preparation of *Escherichia coli* Spiked Urine Samples
Urine Collection and Control Establishment

Urine samples were collected from one individual to create the *E. coli* spiked samples. Urine was collected in a 4L waste container mid-stream immediately upon waking up and brought directly to the lab for use. Before spiking urine with *E. coli*, the untreated urine samples were streaked on an agar plate as a negative control and incubated at 37°C for 48 hours, with observations made at 24-hour intervals. As hypothesized, no bacterial growth was observed post-incubation.

Spectrophotometric Standardization of *E. coli* solutions

To minimize inter-day variability of *E. coli* concentrations, bacterial broth was standardized on the basis of turbidity (0.08 to 0.1 absorbance units) using an absorbance spectrophotometer, with λ = 610nm (Figure 1).[3] Nutrient broth was transferred into a 5mL cuvette using a sterile pipette and zeroed as a blank. Subsequent 5mL cuvettes containing *E. coli* and nutrient broth, which were turbid, were assessed to determine a standard bacterial broth. This standardized solution was then used to spike human urine with *E. coli* to analogue a UTI patient's urine
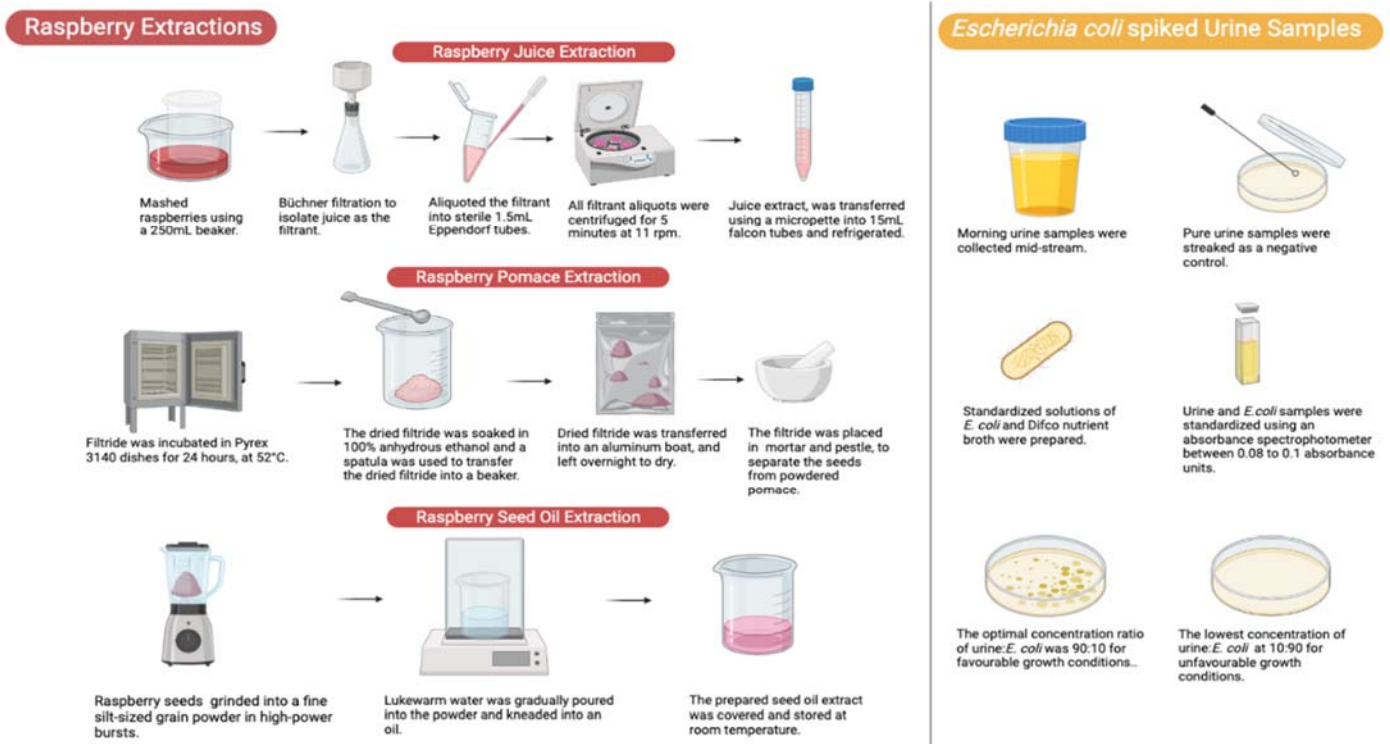
Fig. 1 | Schematic overview of the extraction methods for each of the raspberry components (juice, pomace, seed oil) on the left, and creation of *E. coli* spiked urine samples using spectrophotometric standardization and subsequent determination of the optimal ratio between *E. coli* and urine using streaking techniques.

sample. This standardization process was performed each time a new batch was created.

Determining the optimal *E. coli*:urine concentration

The optimal ratio of *E. coli* and urine for spiking was determined by adding 1mL of standardized *E. coli* to 9mL of urine for a 9:1 ratio.2 A ten-fold serial dilution was then performed in 1.5mL sterile Eppendorf tubes and streaked on agar plates. The streaked plates were left to incubate at 37°C for 48 hours with assessment at both 24 and 48 hours. The optimal concentration ratio, or when the highest bacterial growth was observed, was determined to be 9:1 urine:*E. coli* and was used for all subsequent experiments. Bacterial growth was measured on a scale of zero to four, where zero indicated no growth (negative control) and four indicated maximum growth (positive control).

Extraction Processes of Raspberry Components
Raspberry Juice Extraction

Raspberry juice extraction was achieved by gently mashing store-purchased raspberries using a 250mL beaker inside a Pyrex-3140 plate, until a pulp-like consistency was observed. The mash was transferred to a vacuum Büchner filtration apparatus with the omission of filter paper to prevent seeds from blocking the pores. The mash was stirred using a tablespoon to catalyse the drip process of the filtrant. A plastic pipette was trimmed with scissors to create a wider diameter due to the viscosity of the filtrant, which was aliquoted into sterile 1.5mL Eppendorf tubes and subsequently centrifuged for 5 minutes at 11 rpm to ensure separation of the liquid supernatant and solid pellet. The supernatant (juice extract) was transferred into screw-top vials using a micropipette and stored at 4°C.

Raspberry Powdered Pomace Extraction

The mash filtride, or pomace, from the Büchner filtration was transferred into two Pyrex-3140 glass plates and was homogenized using a tablespoon. The pomace mixture was incubated in an oven at 52°C for 24 hours to remove water. Anhydrous ethanol was used as needed to soak the dried pomace which had strongly adhered to the glass plate. A spatula was used to transfer the soaked pomace clusters into a less adhesive for further overnight drying. The ultra-dried pomace clusters were transferred into a mortar and pestle, and ground gently to form a fine silt-grain sized pomace powder containing solid seeds. A small-pore sieve was used to separate the powdered pomace from the solid seeds, and the latter was covered with foil and stored at room temperature.

Raspberry Seed Oil Extraction

The isolated seeds were ground using a food grinder in rapid, high-power bursts until a sticky seed powder was observed. The seed powder was transferred into a Pyrex-3140 glass plate followed by the addition of 40 mL of warm distilled water. The warm water was gradually added in 1mL increments, using a micropipette, to the seed powder and the mixture was kneaded until a gel-like consistency was observed, and the mixture spread slowly outwards.

Determining the Solvent
Solubility Assay

Since the powdered pomace and seed oil extracts were both in non-liquid physical states, they warranted dissolution in a solvent for serial dilutions and subsequent assays. 10mg of powdered pomace was transferred to a 1.5mL Eppendorf tube and topped up to the 1- mL mark with either 1) 75% ethanol, 2) 95% ethanol, 3) anhydrous ethanol, or 4) 100% DMSO. The tubes were vortexed for seven minutes and the respective solubility of the powdered pomace was observed qualitatively. This was repeated for the seed oil. DMSO showed highest

potential for solubility in both extracts and was determined to be the solvent of choice for performing serial dilutions. DMSO was serial diluted in water, in a 10-fold dilution series, to assess any potential impacts, positive or negative, on bacterial growth as a control, using agar streaking and zone of inhibition. This test determined that no effect was present on *E. coli* growth.

Streaking Techniques

Two main streaking methods were used to assess the antibacterial efficacy of raspberries: normal streaking and zone of inhibition on an *E. coli* agar lawn (Figure 2). Normal streaking was achieved via the use of a sterile inoculating loop that was dipped into the sample of interest. The bacteria were streaked on an agar plate in a zig-zag pattern three times with overlap removing a small amount of sample from the previous streak. This produced three streaks of the sample with decreasing concentration. Between each of the three streaks, the loop was sterilized using a Bunsen burner. This was repeated each time a new agar plate was used. For the zone of inhibition, a lawn of *E. coli* was created on the surface of the agar plate using the previously prepared spiked urine sample. A micropipette was used to add 10μL of the spiked urine sample to an agar plate. A Bacti Cell Spreader was used to evenly disperse the mixture across the entire surface area of the agar plate. A hole punch was used to make 0.5cm filtration paper chads which were placed into each of the 1.5mL Eppendorf tubes of diluted samples. Following centrifugation for 5 seconds, the chads were left to soak for one minute. Agar plates were divided into quadrants to perform a direct side-by-side comparison of the raspberry juice and pomace components at a ten-fold serial dilution with DMSO. Filter papers were removed from the Eppendorf tubes using forceps and placed in the center of each of the corresponding quadrants. The
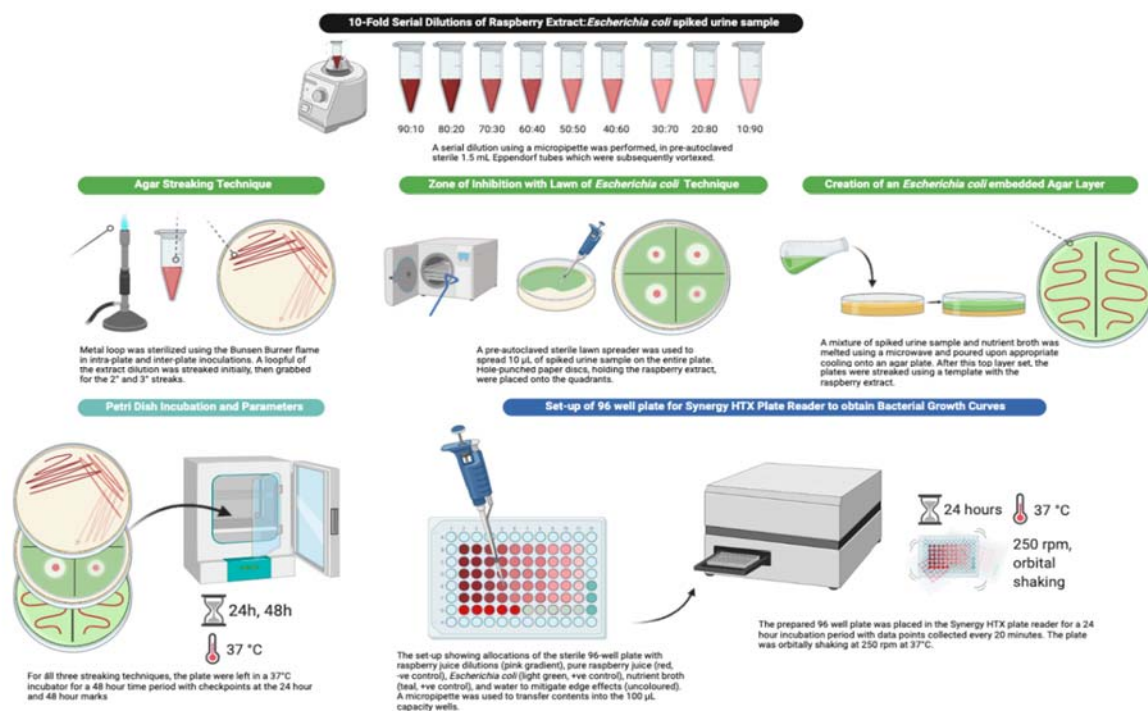
Fig. 2 | Schematic overview of the analysis methods of the raspberry components on the analogue UTI spiked samples, showing the ten-fold serial dilutions performed with the raspberry extract (either juice, pomace, or seed oil), subsequent streaking, lawn, and embedded layer techniques. Also included is a schematic of the 96-well plate set-up for generating a bacterial growth curve.

diameters of all zones of inhibition were measured with a ruler after 24 and 48 hours of incubation.

Bacterial Growth Curves

The efficacy of raspberry juice was determined through a bacterial growth curve performed using a Synergy HTX multi-mode plate reader.[4] A 96-well plate was prepared to conduct the data collection (Figure 2). Five replicates of a 9:1 ten-fold dilution of raspberry juice were assessed. The blank was the nutrient growth media, along with two controls of the pure raspberry juice and spiked urine. The border wells of the plate were filled with 100μL of water to minimize edge effects and conserve heat. The final volume of each well across the entire plate was 100μL. The bacterial growth curve data was obtained over a 24-hour time period with data measurements

collected in 20- minute intervals. The plate reader was set to orbital shaking at approximately 250rpm at 37°C incubation. Initial study results suggest that further experimentation in this area is warranted to properly evaluate the efficacy of raspberry juice through growth curve analyses.

Conclusion

Through a better understanding of the relative location of the PAC content in specific raspberry components, more efforts can be made to make raspberry treatments accessible in the delivery forms of capsules. Their potential as a natural antibacterial agent in tackling UTI infections can be further evaluated through high throughput assays and bacterial growth curves. Through further investigation of proper quantification of the PAC content in the separate raspberry components, the raspberry juice extract showed highest activity as a

home remedy therapeutic for UTIs and warrants further experimentation.

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Exploration of the Anti-Bacterial Properties of Garlic Extract and Streptomycin

Kiran Maheswaran and Sasha Sharma

Antibiotic resistance is an issue becoming increasingly relevant as there is a lack of new antibiotics to treat bacterial strains that have become resistant. This study investigated natural compounds with antibacterial properties as an alternative form of treatment. Allicin is a compound in garlic that is effective against certain drug-resistant bacterial strains. To explore the efficacy of garlic as an antibacterial agent, a methodology involving zone of inhibition testing was developed and the results compared to the antibiotic streptomycin at different concentrations and in combination.

Antibacterial resistance is a phenomenon becoming increasingly prevalent. When bacterial strains evolve to become ineffective against current treatment modalities, infections that were previously treatable can become life-threatening[3]. As a result, it is important to investigate novel compounds with antibacterial properties. One example is allicin, which is an antibacterial compound found in *Allium sativum* (garlic)[4]. The remedial properties of garlic have been recognized throughout ancient civilizations before antibiotics were prevalent in modern society. Today, allicin can be purchased in tablet or capsule form under the brand name Allimax[2].

This study investigated the effectiveness of garlic extract as an antibacterial agent compared to streptomycin, a well-established antibiotic[1]. The investigation was conducted by developing an effective methodology for determining the antibacterial properties of garlic extract by examining the susceptibility of Gram-positive (*Bacillus subtilis*) and Gram-negative bacteria (*Escherichia coli*) by measuring zone of inhibition (ZOI) on agar plates created by different concentrations of garlic and streptomycin, both individually and in combination.

Study methodology was adapted from Palaksha et al.[5] To measure the antibacterial effects of garlic and streptomycin a Kirby-Bauer Test, also known as a zone of inhibition test, was conducted. ZOI testing is a qualitative method that can be used to measure antibacterial efficacy. Petri dishes were prepared for ZOI testing by using a Bacti Cell Spreader to evenly disperse 100µL of bacterial broth solution with a standardized optical density at 610nm of 0.08 to 0.1 absorbance units on a layer of 2.3% agar. The petri dish was then divided into three equivalent sectors and a sterile straw disinfected with 11% Dettol was used as a hole punch to create one hole in the center of each of the three sectors and one additional hole in the center of the dish. Each of the hole punched cylindrical pieces of agar were removed using forceps that had been sterilized via heating in a Bunsen burner. Finally, a 20µL volume of each concentration of test solution was added to their designated hole with the center hole acting as control with 20µL of distilled water (Figure 1a). The test solutions were 25, 50, and 100% garlic extract; 1, 10, and 25mg/mL streptomycin; or 25% garlic extract with 1mg/mL streptomycin and 50% garlic with 10mg/mL streptomycin. The agar plates were incubated at either 37°C for *E. coli* or 30°C for *B.*
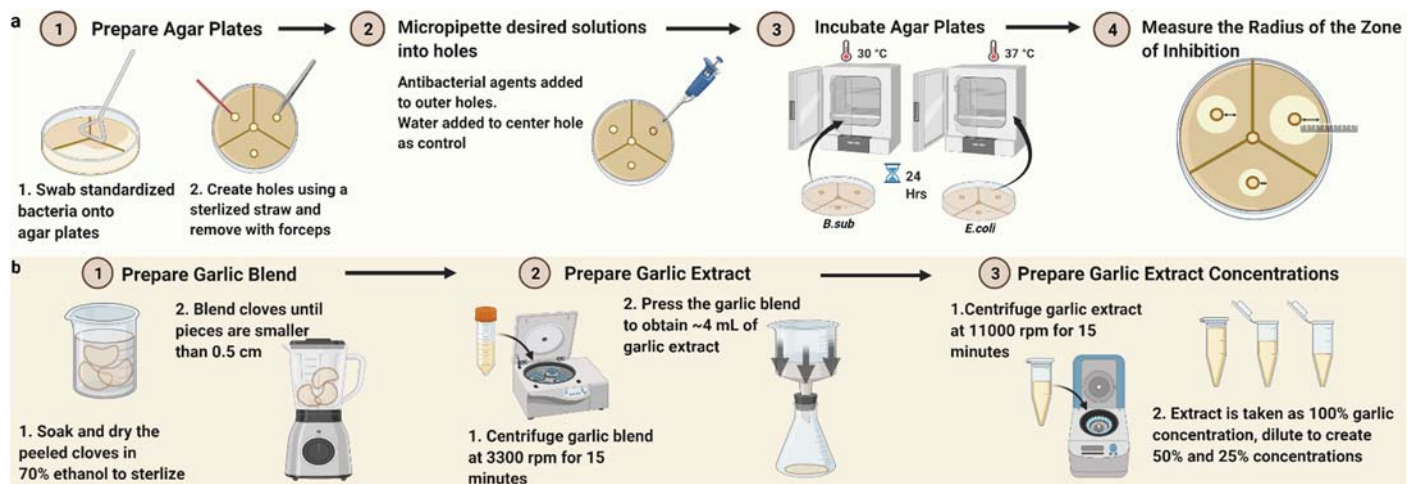
Fig. 1 | Flow chart depicting the experimental procedure and the methodology for preparing the garlic extract concentrations. a, Step 1 involved preparing the agar plates by dividing them into three sections, swabbing them with standardized bacterial strains, and creating four holes using a sterile straw. The residual agar gel was removed using sterile forceps. Step 2 involved pipetting the antibacterial agents and control into the plate. In step 3, the plates were incubated for 24 hours. In step 4, the ZOI was measured using a ruler. **b,** In Step 1 the garlic blend was prepared by sterilizing the cloves. Followed by blending the cloves. In Step 2, the garlic extract was prepared by centrifuging the blended garlic at 3300rpm for 15 minutes, then pressing the garlic to extract the liquid. In Step 3 the garlic extract was centrifuged, then the supernatant was used as the 100% garlic extract concentration, which was then diluted to create 50% and 25% concentrations of garlic extract.

subtilis for 24 hours after which the ZOI around each of the holes was determined by measuring from the edge of the hole to the outer edge of the zone of no bacterial growth (Figure 1a).

Powdered streptomycin (CAS: 3810-74-0) was diluted with distilled water to create solutions of 1, 2, 10, 20, and 25mg/mL streptomycin. The 2 and 20 mg/mL solutions were used in the combination treatment to account for the streptomycin solution concentration being halved when equal volume of garlic solution was added. The creation of the garlic extract was done using store-purchased Chinese garlic. The garlic cloves were peeled and soaked in 70% ethanol for 30 seconds to disinfect their surface, then left to dry in a fume hood for 5 minutes. The cloves were then placed in a blender (Magic Bullet brand) and blended until most pieces were smaller than ~0.5cm. The garlic pieces were placed in 15mL conical tubes and centrifuged at 3300rpm for 15

minutes. The extract was separated from the blended garlic pieces with a Büchner funnel mounted on a 250mL Erlenmeyer flask which used a beaker as a press (Figure 1b). This resulted in the liquid garlic extract being collected into the Erlenmeyer flask. Approximately, 4mL of crude garlic extract was obtained from one whole garlic. A 3mL portion of the crude extract was aliquoted into two 1.5mL Eppendorf tubes and centrifuged at 11000rpm for 15 minutes. The supernatant was considered to be 100% garlic extract and was stored in clean 1.5mL Eppendorf tubes. Subsequent dilutions were performed using distilled water as the diluent to create solutions of 25 and 50% garlic extract.

Two trials were performed for each of the individual concentrations with each bacterium, and one trial was performed for each of the combination treatments with each bacterium.
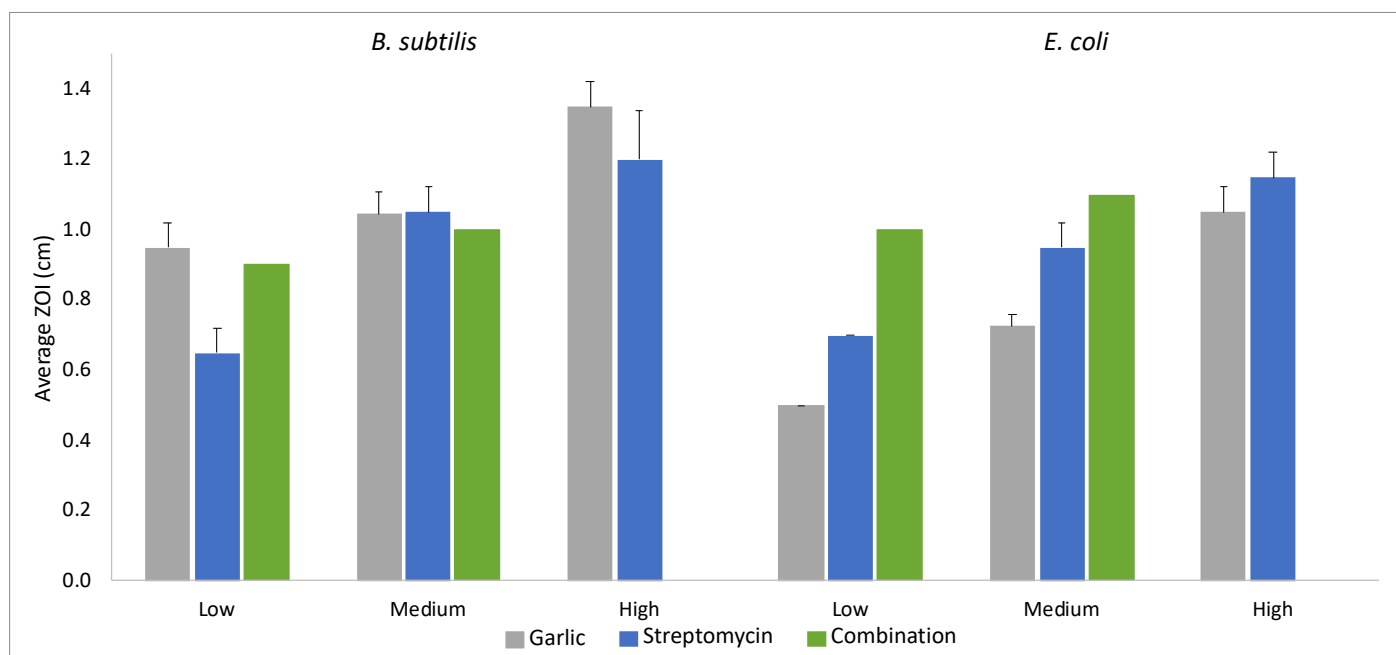
Fig. 2 │ Average ZOI when testing different concentrations of garlic and streptomycin in combination and individually. The bars show the average ZOI of garlic (grey), streptomycin (blue), and combination of garlic and streptomycin (green) against both *B. subtilis* (left) and *E. coli* (right). Error bars shown are standard deviation (n=2), not included for combination treatment due to only having one trial. For streptomycin, the concentrations tested were 1 (low), 10 (medium), and 25mg/mL (high). For garlic it was 25 (low), 50 (medium), and 100% (high). For the combination treatments 1mg/mL with 25% was low and 10mg/mL with 50% was medium. Garlic is shown to be more effective against *B. subtilis* than *E. coli*, while streptomycin and the combination treatments had similar effects on both *B. subtilis* and *E. coli*.

The results (Figure 2) show that streptomycin was found to have very similar inhibitory effects on *B. subtilis* and *E. coli*. This is consistent with streptomycin's capability of targeting broad spectrum bacterium. However, garlic extract was found to be more effective against *B. subtilis* compared to *E. coli*. The combination treatments were slightly less effective than using garlic alone when applied to *B. subtilis*. However, when the combination treatment was used on *E. coli* it was found to be more effective than garlic or streptomycin alone.

These results suggest that garlic may be more effective against gram-positive bacteria like *B. subtilis*. Future studies should be done to explore the effect of garlic against different Gram-positive bacteria to determine its broader efficacy. Gram-positive bacteria do not have multiple outer membranes, making it easier for certain antibacterial agents to enter the cell. Additional combination treatment studies could be conducted to examine whether the introduction of garlic to an antibiotic treatment plan can enhance its effects. Allicin and streptomycin differ in their modes of entry into the cell and in their mechanisms of action[1,4]. Allicin is a lipophilic molecule that can readily pass through the lipid membrane, while streptomycin requires ATP-channels[1,4]. Additionally, streptomycin targets protein synthesis, while allicin targets DNA, RNA and protein synthesis as well as inhibiting enzymes and damaging the cell membrane[1,4]. The interaction between the two can potentially enhance the overall antibacterial capabilities, which may be what caused the increased effect of the combination treatment on

E.coli in this study. A potential study could examine the efficacy of the tablet form of allicin that can be purchased over the counter. It should be noted that the presence of allicin within the garlic extract was assumed but not confirmed in this study. Using a concentrated form of allicin can determine with greater confidence that the results are caused by allicin. Similarly, allicin detection methods such as using mass spectroscopy could be performed.

The applications of this study can assist in the advancement of using natural products like allicin to treat bacterial infections.

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Investigating Bacteriophage Presence in Soil and its Effects on Escherichia coli, Bacillus subtilis, and Micrococcus luteus

Katherine Dalkie and Yahya Jama

Bacteriophage therapy has been a growing field of treatment for bacterial infections for the past century. However, little progress has been made in the isolation and characterization of phages. In past studies, researchers have tried to isolate phages from soil samples with limited success in this study soil isolation techniques were developed to yield enriched phage plaques. This was done by plating a cultured phage sample, as well as one isolated from the environment, to observe their inhibitory effect on bacterial growth.

Bacteriophages, also known as phages, are viruses which can infect bacteria. Soil has its own incredibly diverse microbiome, including bacteria and phages. Some studies estimate there could be billions of phages in just one gram of soil[1]. As a result, soil environments provide a vast number of phages to be isolated for a specific host bacterium.

Additionally, phages can be highly specific to certain strains of bacteria and thus infect and lyse them. The host specificity that phage have often make them a great candidate to be used in phage therapy to treat a bacterial infection, especially in fighting antibiotic resistant bacteria. However, while there is ample research on bacteria there are few studies that characterize phages.

Previously, various soil phage isolation protocols have had very low success rates of phage isolation[2]. These early methods often relied on adding toxic agents to soil in hopes of giving rise to phage-specific bacterial strains. Such methods had little efficacy and are impractical for widespread isolation of phage from soil. The purpose of this study was to develop a straightforward technique to isolate and enrich phage from soil samples. The premise of this study was to determine if isolated phage samples from soil are able to inhibit bacterial growth on *Bacillus subtilis*, *Micrococcus luteus,* and two strains of *Escherichia coli: E. coli BL12* and *E. coliG*, the latter an in-house wildtype strain of unknown origin. Through a process of purification and enrichment followed by testing, the goals were to determine inhibition of bacterial growth thereby proving the presence of phages, and test for growth inhibition of various phage-bacteria combinations.

Methodology

Bacteria Liquid Culture Preparation

To prepare bacteria cell cultures, the bacteria strains *E. coliG, E. coli BL21, M. luteus,* and *B. subtilis* had each of their colonies picked from cultures on agar plates using a sterile inoculating loop and submerged into separate sterile Erlenmeyer flasks each containing Luria Broth (LB). The flasks with *E. coli* and *M. luteus* cultures were grown at 37°C for 24h while *B. subtilis* was grown at 30°C for 24h.

Soil Sample Purification

To isolate phage from a soil sample (Figure 1), the methods developed by Cross et al.[2] and The College of Natural Science[3] were used. First, a soil sample was collected in a Ziploc bag from the McMaster
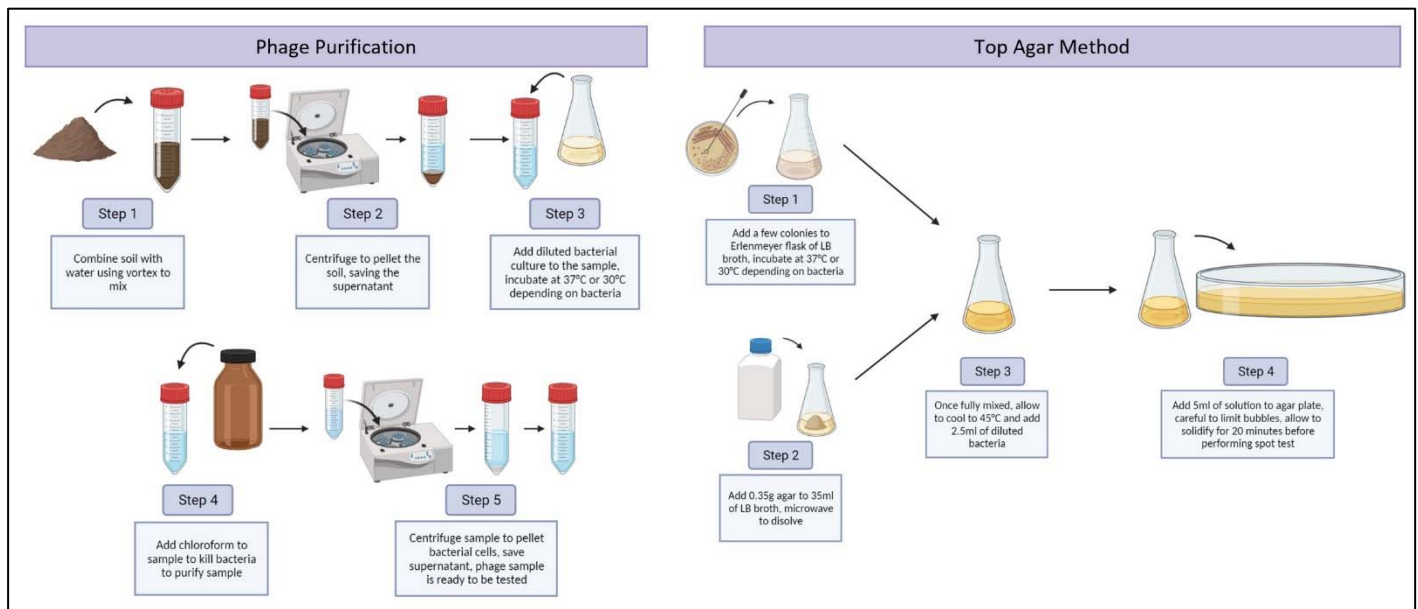
Fig. 1|Flow chart of phage isolation (left) and top-agar (right) methods. The phage purification starts with creating a soil suspension with distilled water using a vortex, followed by centrifugation at 3600rpm for 30 minutes, after which the supernatant is removed. Bacterial culture is added to the sample then incubated overnight at 37°C for *E. coli*, *M. luteus* and 30°C for *B. subtilis*. After incubation, chloroform is added to the solution to kill bacteria, followed by centrifugation for 90 minutes at 3600rpm and the resulting supernatant is a concentrated phage sample. The top agar method first creates bacterial culture from plated bacteria. Agar is added to LB broth then microwaved in 15 second intervals to dissolve the powder. Once the mixture cools to 45°C, bacterial culture is added, resulting in a solution of 7g/mL of agar. The mixture is then layered on top of a pre-poured agar plate and allowed to solidify before a spot test is performed.

University Greenhouse in an area that was shady, moist, and near a *Phyllostachys Aurea* (Golden Bamboo) plant. In the lab, 14.2g of the soil sample was transferred to a 50mL conical tube, followed by the addition of 35mL of distilled water. The tube was vortexed for 5 seconds to create a soil suspension followed by centrifugation at 3600rpm for 30 minutes to pellet the bacteria and soil. The supernatant was then transferred to a new sterile conical tube.

The Bacteriophage Enrichment Step
The methods to propagate the bacteriophage were adapted from Sambrook and Russel[4] to allow the bacteriophage to proliferate, producing a solution with a high concentration of phage. To create the broth needed to propagate the phage, 2mL of diluted bacterial culture was added to 8mL of liquid phage sample from the soil and 1mL of phage buffer

(150mM NaCl, 40mM Tris-Cl, 10mM $MgSO_4$, 1mM $CaCl_2$) and 35mL of LB broth in a 50mL tube. This step was repeated for each bacterial strain, resulting in 3 tubes each with a different bacterium-enriched phage. A spectrometer was used to record the absorbance of each bacteria culture prior to adding it to the broth. Contents of the test tubes were then poured into separate sterile Erlenmeyer flasks and then incubated in water baths (37°C for *E. coli* and *M. luteus* and 30°C for *B. subtilis*) for 5 hours with agitation every 15 minutes.

Bacteriophage Isolation from a liquid lysate
The flasks were removed from the water baths and their contents were poured into separate sterile conical tubes. Next, 40μL of chloroform was added to the 3 bacteria-enriched phage samples to kill bacteria resulting in a purified phage sample. The tubes were
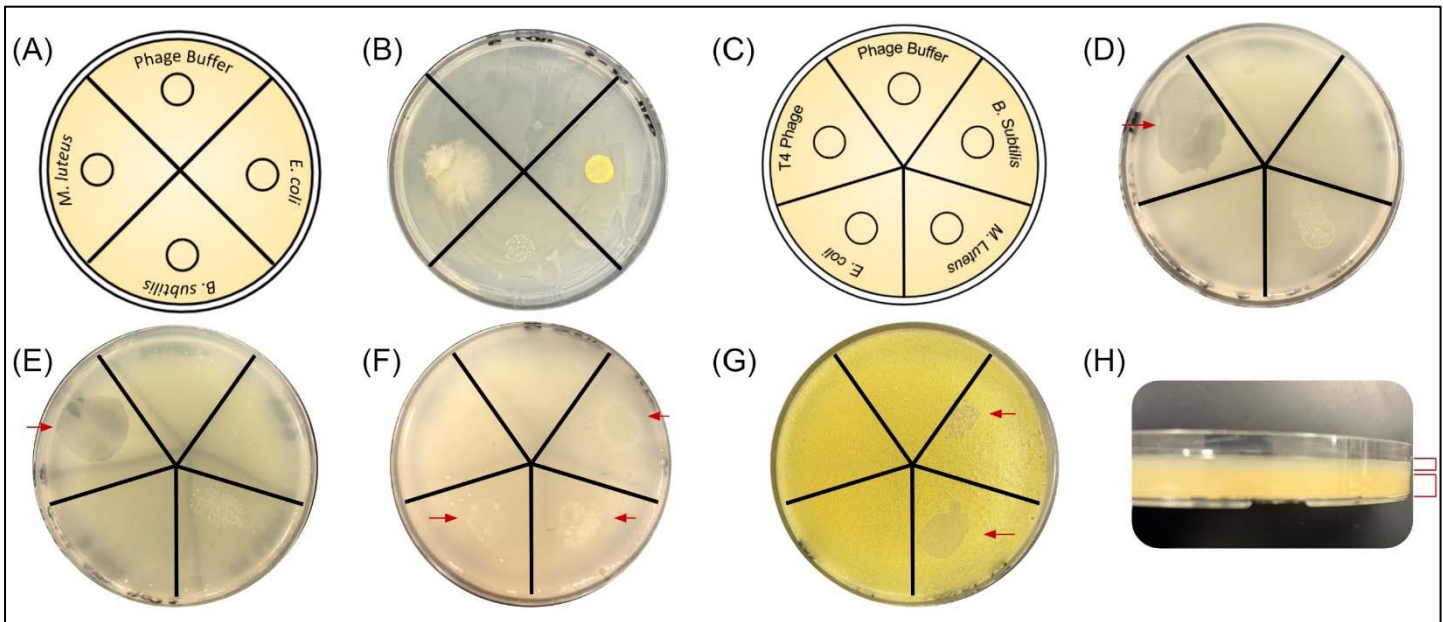
Fig. 2|Agar Plates. A and C outline the layout of B and D-G respectively, with the enriched phage samples for each bacterium, the phage buffer used as a negative control, and the pure T4 phage sample.
(B) *E. coliG* plate prepared by standard lawn method and did not yield inhibition of bacterial growth and the spots appeared to contain contaminants. (D-G) *E. coliG*, *E. coli BL12*, *B. subtills*, and *M. luteus*, respectively, were grown using top-agar method, and inhibition of growth was observed on each plate, as noted by the red arrows. (F) Side view image of a plate prepared by the top-agar method. As seen, the top layer has a lighter colour due to the lower agar concentration.

gently stirred and left to incubate at room temperature for 10 minutes then placed into a centrifuge for 90 minutes at 3600rpm. After they were spun down, the supernatant from each tube was added to a new labeled conical tube and stored at 4°C.

Standard Agar Plate Preparation

Four bacteria agar plates were prepared using the standard agar plate preparation technique, described by Cross et al[2]. To prepare them, each plate was designated one of the 4 bacterial strains and was divided into 5 separate labeled sections (Figure 2A,C), one for the positive control T4 sample, one for the phage buffer as the negative control, and one for each of the three phage samples. Next, on each plate 110µl of *E. coliG, M. luteus*, *B. subtilis* and *E. coli BL21* culture were pipetted on each plate and then distributed evenly using a Bacti Cell Spreader.

Bacteria Culture Dilution

Standardized bacterial culture was required to produce the agar plates that were prepared using the Top Agar method. First, the 4 bacterial stock cultures (*E. coliG*, *E. coli BL21. M. luteus* and *B. subtilis*) were diluted to an absorbance between 0.08 to 0.10AU at 610nm determined by a SPARK device spectrometer. Initially, 10mL of broth was pipetted into a labeled sterile colonial tube, then 1mL of bacterial culture was pipetted into the tube. After mixing, 5mL of the diluted solution from was pipetted into a cuvette which was placed into a spectrometer. If the OD was below the range more culture was added, and if it was above the range more LB broth was added. This process was repeated until the OD was measured within the desired range. This was done for each culture, resulting in 4 tubes each containing one of the diluted bacterial cultures with OD between 0.08 and 0.1AU.

Top Agar Plating

In this agar plate preparation method, 70mg of Difco Nutrient Agar and 9mL of LB broth were added to labelled Erlenmeyer flasks. Kimwipes were used to cover the flask openings before being heated in a microwave. The flasks were pulsed for 15-second intervals and swirled after each interval until all the agar powder was dissolved in the LB broth. Sterile thermometers were used to monitor agar temperature until 45°C was obtained, at which point 1mL of the diluted bacteria culture was pipetted into the flask and swirled to mix. A 5mL aliquot of this solution was pipetted into a pre-poured agar plate labelled with 5 separate sections for the test phage samples (Figure 2A,C). The plate was gently swirled until the agar was evenly spread across the surface and free of bubbles. This process was repeated for all 4 bacteria strains. Before proceeding with further steps, the agar plates cooled and solidified for 30 minutes

Spot Test

Phage samples were retrieved from the fridge and placed in a 37°C water bath for 5 minutes to warm up. Next, 10µl of each phage sample and buffer was added to its corresponding labeled section of one of the 8 agar plates (4 plates with standard lawns, and 4 prepared by top-agar). The plates dried for 25 minutes before placing them in the incubator overnight at their specific temperatures (37°C for *E. coli* and *M. luteus* and 30°C for *B. subtilis*).

Tris-HCl Solution

To create a Tris-HCl solution, 3.025g of tris powder was dissolved in 10mL of distilled water. Next, 1.5mL of HCl was pipetted into the solution with 0.1mL of HCl being added incrementally thereafter. After every 0.1mL addition the pH was tested to determine whether the pH of the solution was within the desired range of pH 7-8. The desired pH range was reached after adding 1.7mL of HCl which resulted in a 1M solution. This stock solution was further diluted with distilled water to create a 0.1M Tris-HCl solution.

Phage Buffer Solution

A phage buffer control was created to be used whenever phage was either plated or mixed with bacteria. Additionally, it also served as the control throughout the experiment. The sample consists of 150mM NaCl, 40mM Tris-Cl of pH 7.4, 10mM $MgSO_4$, 1mM $CaCl_2$, making 250mL of solution. To prepare the solution. 200mL of distilled water was added to a 500mL beaker with a stir rod along with 2.19g NaCl, 0.617g MgSO4 heptahydrate, and 36mg of CaCl dihydrate. Afterwards 10mL of the Tris-HCl solution was added.

Results

Initial studies utilized a purchased lyophilized lambda phage for *E. coli* host strain W3110 from Sigma Aldrich, which was prepared and tested against *E. coliG* in an agar plate. The lambda phage had no visible effect on the growth of the bacteria, which was likely due to incompatibility between phage and bacterial strain. The specific host strain was not available, and *E. coliG*, was used in place due to availability. After this study, the focus of the experiment shifted towards sourcing phages naturally from soil.

Additionally, the first trial of isolating phage from the soil sample did not produce the expected results. At this point, the phage was likely in too low of concentration to inhibit bacterial growth. This was corrected in subsequent studies through the addition of an enrichment step by introducing bacteria to the phage sample in order to propagate the phages.

The top agar method produced more reliable and quantifiable results compared to the standard lawn plating method. This was due to the uniform growth that allowed for a clearer visual of growth

inhibition. Additionally, the lower density medium this method provided (Figure 2H) allowed the phage to diffuse and infect other cells.

The first trial after introducing the enrichment step resulted in contaminants on the plate (Figure 2B). The likely cause was due to insufficient purification, and thus an additional purification by centrifuging was done.

The experiment revealed accurate bacterial growth by phage through the use of the T4 phage sample and confirmed that the previous studies had not been due to phage inhibition. The T4 phage had the largest area of inhibition and was effective against the two strains of *E. coli* (Figure 2D,E). The phage solution enriched with *M. luteus* had inhibited the growth of both *B. subtilis* and *M. luteus* (Figure 2F,G). Similarly, the phage solution enriched with *B. subtilis* inhibited the growth of both *M. luteus* and *B. subtilis* (Figure 2F,G). Finally, the phage solution enriched with *E. coli* only inhibited the growth of *M. luteus* (Figure 2G). The soil-derived phage solutions resulted in inhibition across multiple bacterial strains, suggesting either a more general specificity, or multiple phages present.

This study illustrates a clear method in which phages can be isolated from soil samples and can be used to have an inhibitory effect on bacterial growth. Additionally, the presence of phages in soil by this investigation can provide an avenue to discover different phages for phage therapy.

References

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Acknowledgements