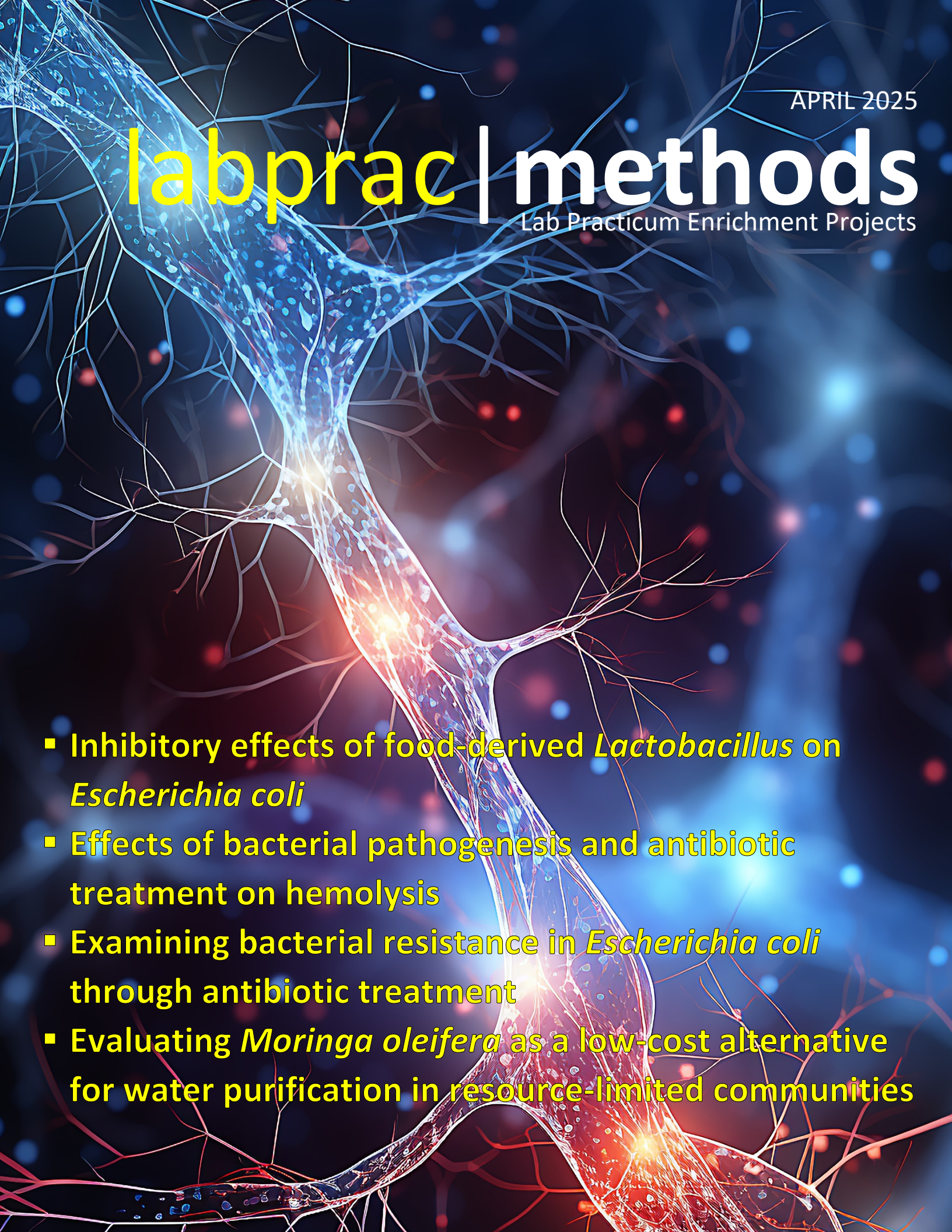

APRIL 2025

labprac | methods

Lab Practicum Enrichment Projects

- Inhibitory effects of food-derived *Lactobacillus* on *Escherichia coli*
- Effects of bacterial pathogenesis and antibiotic treatment on hemolysis
- Examining bacterial resistance in *Escherichia coli* through antibiotic treatment
- Evaluating *Moringa oleifera* as a low-cost alternative for water purification in resource-limited communities

labprac|methods BRIEF COMMUNICATION

Inhibitory Effects of Food-Derived *Lactobacillus* on *Escherichia coli*

Caroline Bazydlo and Alexandra Biernacki

Antibiotic-resistant infections pose a rapidly increasing health risk, however, many probiotics exhibit antimicrobial properties against pathogenic bacteria. In this study, the efficacy of lactic acid bacteria (LAB) as an inhibitor of *Escherichia coli* growth was assessed through a three-phase approach. In Phase 1, pure *Lactobacillus acidophilus* was co-cultured with *E. coli* to establish a baseline bacterial interaction. In Phase 2, fermentation starter kits with minimal ingredients were cultured as a bridge between pure LAB and probiotic foods. In Phase 3, probiotic-rich foods including kimchi, sauerkraut, kombucha, and yogurt were tested. All phases utilized data collected from kinetic growth absorbance assays and Gram-stained slides. This study found synergistic effects of coculturing LAB and *E. coli*, with implications for future studies utilizing LAB as an antibacterial agent.

Antibiotic resistance is a major global health challenge, with projections estimating over 39 million direct deaths by 2050 as common infections and minor injuries will again become deadly.[1] A potential solution is lactic acid bacteria (LAB), which naturally occurs in fermented foods, and has demonstrated abilities to inhibit growth of pathogenic bacteria, through the production of lactic acid, which lowers the pH of the surrounding environment.[2]

Methods

Research was conducted in three phases using methodology summarized in Figure 1. Phase 1 used pure *Lactobacillus acidophilus* (Custom Probiotics Canada) to determine a baseline for LAB and *E. coli* interaction. Phase 2 used fermentation starter kits to investigate the impacts of added ingredients on the bacterial interaction. KIT1 (NP Selection) contained *Lactobacillus bulgarius, Lactobacillus salivarius, Streptococcus thermophilus,* and *Lactobacillus reuteri*, while KIT2 (Yogourmet) contained *Lactobacillus bulgaricus, Streptococcus thermophilus, Lactobacillus acidophilus*, maltodextrin, and sugar. In Phase 3, LAB was isolated from probiotic-rich foods including kimchi, kombucha, sauerkraut, and yogurt, which presented confounding variables such as effects of pH. The phases allowed for increasing complexity and applicability, while maintaining reliability of results.

Preparation of LAB Cultures

Tryptic soy broth (TSB) cultures were conducted in 25mL of broth for 48h at 35°C, while agar cultures were conducted on TSB agar plates and incubated for 72h at 35°C. In Phase 1, 0.1g of pure *L. acidophilus* was cultured in TSB broth. In Phase 2, 2g from each fermentation kit were individually cultured in TSB, then streaked and cultured on agar. Afterwards, visually distinct target colonies were recultured in TSB (Figure 1 – top row) in order to create pure LAB cultures. Phase 3 followed the same culturing protocol as Phase 2, using 10g each of kombucha, kimchi, yogurt, and sauerkraut.

Individual target colonies from agar plates were transferred to slides for subsequent staining and microscopic observation. In Phase 1, one bacterial type was identified. In Phase 2, one bacterial type was identified in Kit 1 denoted KIT1, while two were identified in KIT 2 and denoted as KIT2A and KIT2B. In Phase 3, single bacterial types were identified in kimchi, kombucha, and sauerkraut denoted KIM, KOMB, and SKT respectively, while two bacterial

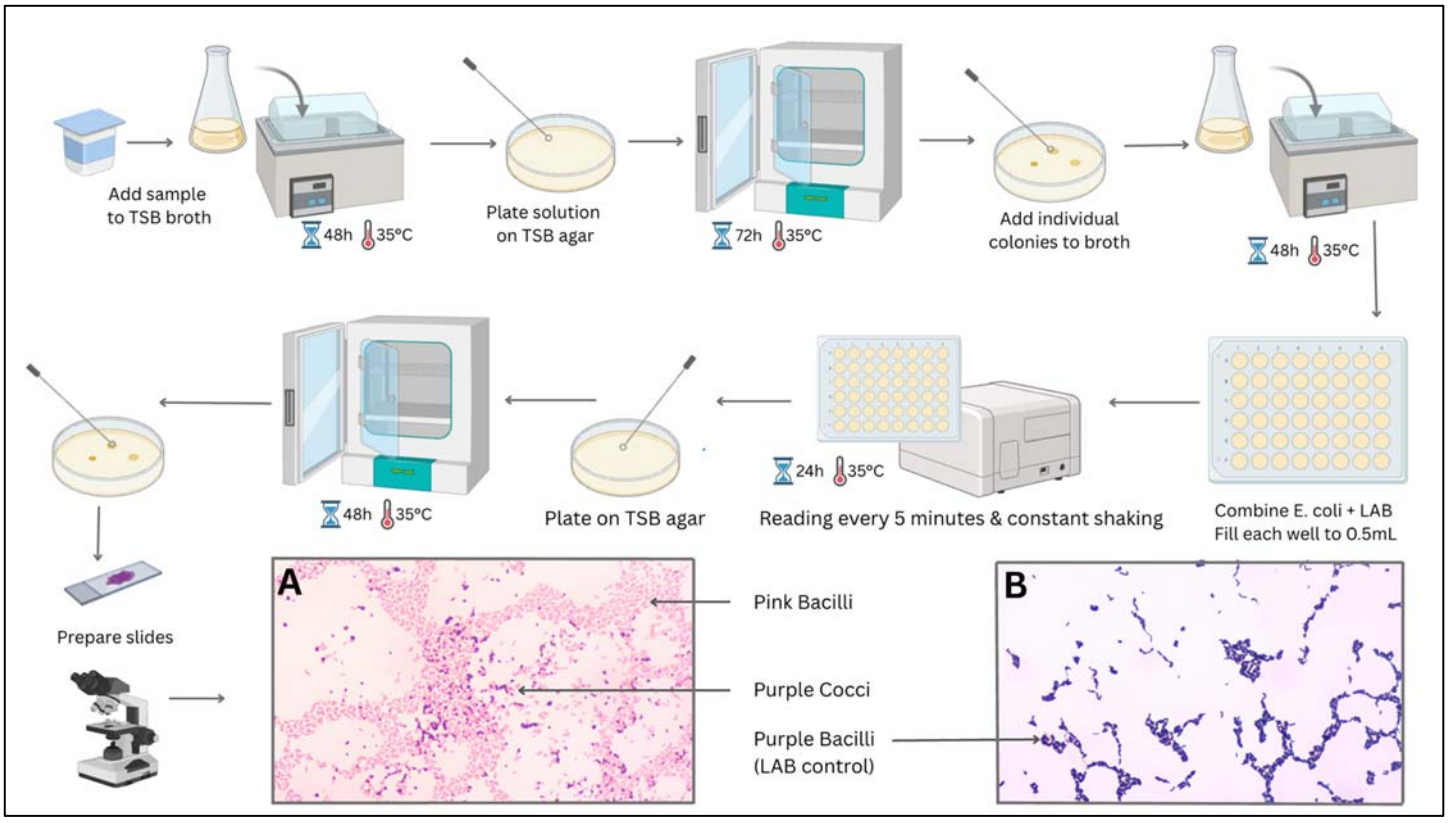

Fig. 1 | Schematic overview of methodology used to culture and test LAB with *E. coli.*

types were identified from yogurt, denoted YOG1 and YOG2.

Kinetic Growth Curve Assay Set Up

For each phase, final cultures of pure LAB and *E. coli* were standardized in TSB. A 48-well plate was used to assess the growth of 0.5mL LAB and *E. coli* solutions at concentrations of 95:5, 80:20, 65:35, 50:50, 35:65, and 20:80, along with controls (media only, *or E. coli* in media). A kinetic growth curve (KGC) test was conducted using a Synergy HTX multi-mode plate reader, set to 35°C with constant shaking, measuring absorbance at 5-minute intervals for 24h.

Post-Kinetic Growth Curve Analysis

Upon completion of the KGC, samples at each concentration were recultured on TSB agar plates and incubated to conduct further analysis on bacterial growth. Slides were prepared by plucking colonies from the agar plates and placing them in drops of distilled water on glass slides. After drying, the slides were Gram stained and coverslipped. Since both LAB and *E. coli* have similar morphology, staining was used to differentiate between the two. LAB is predominantly gram-positive, while *E. coli* is gram-negative, staining them purple and pink respectively.[3] In Figure 1, slide image A depicts a combination of LAB and *E. coli* where the light pink rod shapes are *E. coli* and dark purple cocci shapes are LAB. Image B depicts a LAB control slide with dark purple rod-shaped LAB.

To analyze the KGC absorbance data, the repetitions of each test condition were averaged for every data point. The difference between average absorbance at each point and the initial average absorbance was subsequently plotted. For all tests, the positive control (POS) exhibited greater absorbance than the negative control (NEG).

Phase 1 Results (Figure 2 – 1A & 1B)

L. acidophilus combined with *E. coli* demonstrated an earlier and more rapid growth increase compared to pure *L. acidophilus* in all test conditions. LAB

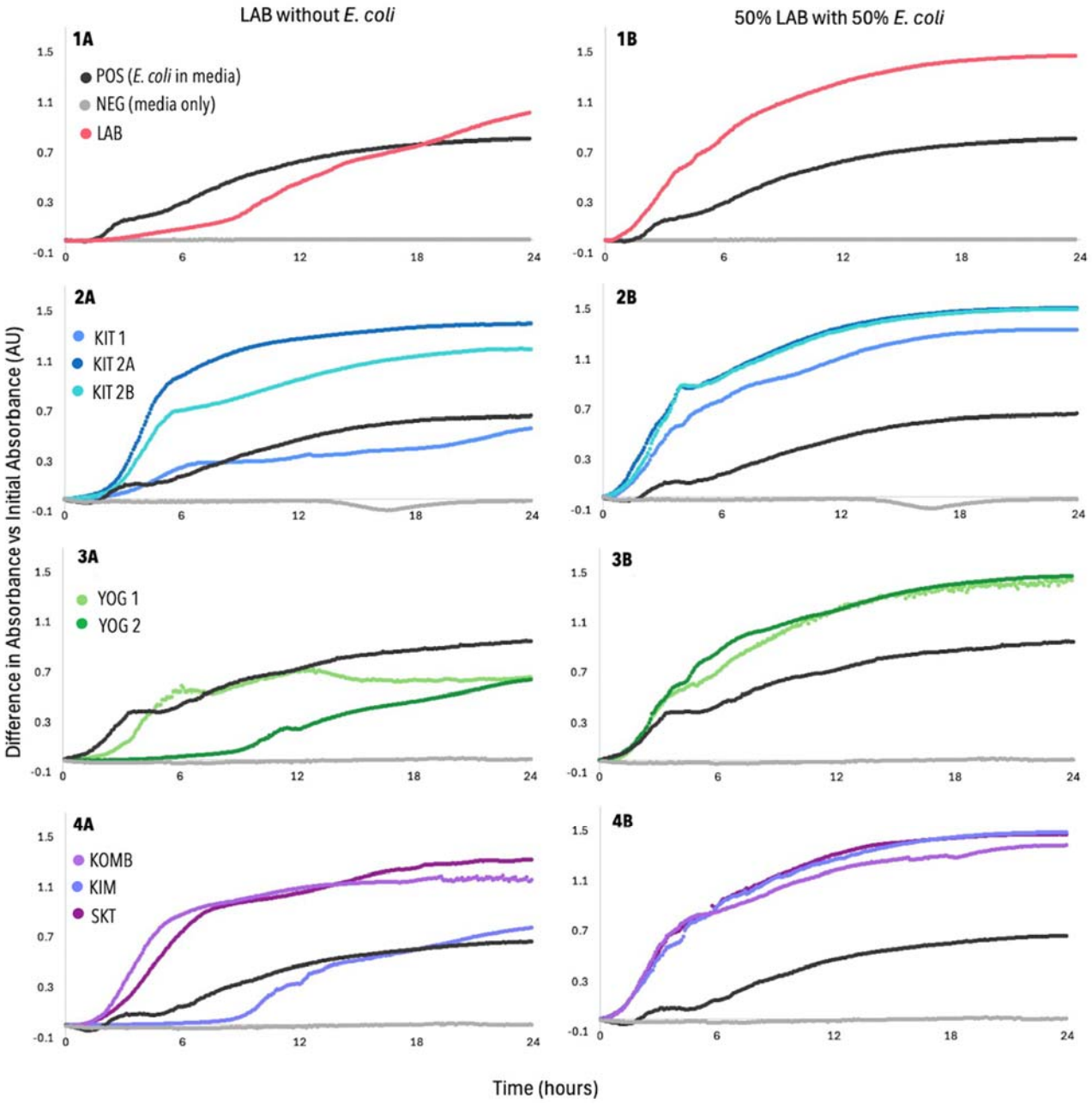

Fig. 2 | Summary panel of 24-hour kinetic growth curves based on absorbance at a wavelength of 600nm measured using a plate reader.

combined with E. coli (panel 1B) reached a maximum absorbance of 1.4AU, greater than the absorbance of either LAB (1.1AU) or E. coli (0.7AU) alone (panel 1A). Samples ranging from 65-35% LAB had faster growth upticks and overall growth than the POS control, with minimal variation, exemplified by the 50:50 trial.

Increased absorbance when LAB was combined with *E. coli* is suggestive of synergistic growth. Microscopic analysis revealed similar morphology between LAB and *E. coli*, necessitating further analysis to determine bacterial identification.

Phase 2 Results (Figure 2 – 2A &2B)

In all test conditions, KIT1 combined with E. coli showed increased overall growth compared to its control, indicated by the maximum absorbance

increasing from 0.4AU (panel 2A) to 1.2AU (panel 2B). The 65-35% LAB tests had the most growth, indicating a synergistic effect in this range. Test conditions of KIT2A followed the growth pattern of its control, and maximum absorbance remained at 1.4AU. When combined with *E. coli,* KIT2B growth increased, indicating synergy, and interestingly mirrored the KGC pattern and overall growth of KIT2A. Microscopic analysis found purple cocci in all Phase 2 controls, and a combination of these cocci and pink bacilli in test conditions, confirming the presence of LAB and *E. coli,* indicating synergy.

Phase 3 Results (Figure 2 – 3A, 3B, 4A, & 4B) In control conditions, YOG1 showed faster initial growth, and a sooner plateau compared to YOG2; however, both ended about 40% lower than the *E coli* control at 0.5AU (panel 3A). In the 95% LAB trial, YOG2 maximum absorbance reached 1.4AU, indicating that overall growth more than doubled, which remained similar for all trials (panel 3B). YOG1 overall growth increased slower and was nearly identical to YOG2 in the 65-35% LAB range (panel 3B), after which it began to decline again. A synergistic effect is hypothesized between E coli and YOG bacteria (YOG2 at all concentrations and YOG1 between 65-35% LAB). Microscopic analysis, showed bacteria with morphology characteristic of *E. coli* and LAB, further supporting a synergistic effect.

The KIM control showed little growth until 9h, and the least overall growth compared to SKT and KOMB (panel 4A). However, in the 95% LAB trial, KIM growth spiked before the 3h mark and became progressively sooner as *E. coli* concentration increased (panel 4B), indicating a positive correlation between *E. coli* concentration and growth speed. KIM growth was greatest between 65-35% LAB with a maximum absorbance of 1.4AU, suggesting the highest synergistic effect in this range. Microscopic analysis revealed cocci in KIM controls, but bacilli and cocci in KIM test conditions, indicative of LAB and *E. coli* in peak growth conditions, supporting synergy. SKT and KOMB growth spiked sooner in test conditions, but overall growth remained similar to the respective, indicating minimal effect on bacterial growth. Microscopic observations revealed similar morphology in SKT and KOMB bacteria compared to *E. coli*, prompting no further conclusions.

Conclusion

Macroscopic and microscopic observations demonstrate that LAB and *E. coli* co-culturing affects bacterial growth. A large increase in absorbance was seen in trials with pure LAB, KIT1, YOG1, YOG2, and KOMB at a 1:1 ratio with E coli, suggestive of a synergistic effect. Pure LAB, KIT1, and YOG2 all had the most synergy when combined at 65:35, 50:50, and 35:65 ratios with *E. coli*, prompting interest in further exploring this range. A small increase was also observed in trials with KIT2B and SKT, while the absorbance remained relatively similar for KIT2A and KIM, suggesting minimal effects on growth in these test conditions. Slide analysis revealed diverse bacteria in all test conditions and in some control conditions, which implies synergy between bacterial species. The methodology was able to successfully isolate LAB from fermentation kits and probiotic-rich foods, as demonstrated by similar KGC patterns to pure LAB. Additionally, the analysis identified large differences between control and test trials, providing insight into target bacterial interactions. Study results demonstrated a variety of interesting effects of LAB on *E. coli*, prompting interest in further studies.

References

1. Naghavi, M. *et al.* Global burden of bacterial antimicrobial resistance 1990–2021: a systematic analysis with forecasts to 2050. *The Lancet* **404**, 1199–1226 (2024).
2. Atassi, F. & Servin, A. L. Individual and co-operative roles of lactic acid and hydrogen peroxide in the killing activity of enteric strain Lactobacillus johnsonii NCC933 and vaginal strain Lactobacillus

gasseri KS120.1 against enteric, uropathogenic and vaginosis-associated pathogens. *FEMS Microbiol. Lett.* **304**, 29–38 (2010).
3. Szkaradkiewicz, A. K. & Karpiński, T. M. Probiotics and prebiotics. *J. Biol. Earth Sci.* **3**, M42–M47 (2013).

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labprac|methods BRIEF COMMUNICATION

Effects of Bacterial Pathogenesis and Antibiotic Treatment on Hemolysis

Oviya Sathiyanarayanan and Jenny Yong

Bacterial-induced red blood cell damage, or hemolysis, can be treated with antibiotics. However, specific bacterial-antibiotic interactions remain understudied. This study investigated the effect of streptomycin, tetracycline, gentamicin, and vancomycin on hemolysis mitigation caused by *Bacillus subtilis*, *Escherichia coli*, and *Micrococcus luteus*. The plasma of sheep blood cultures containing drug and bacteria-broth concentrations were examined with an endpoint absorbance reading to evaluate hemolysis. Tetracycline treatments produced greater absorbance values than the controls. Hemolytic activity was further assessed using blood agar plates. Hemolysis was seen in *B. subtilis* and *E. coli*.

The destruction of red blood cells (RBCs), also known as hemolysis, can result from bacterial infections. Bacterial hemolysins are virulence factors that lyse RBCs by forming pores in the cellular membrane.[1] Strains such as *Escherichia coli* and *Bacillus subtilis* are known to have hemolytic activity.[2,3] Pathogenesis can be mitigated via antibiotic treatment, yet higher levels of certain antibiotics can exacerbate hemolysis.[4] Altogether, specific aspects of blood-bacteria interactions remain understudied. Understanding this can improve treatment efficacy and knowledge of hemolytic pathogenesis.

This study compared the effect of streptomycin and tetracycline on hemolytic behaviour of *B. subtilis* and *E. coli* using absorbance-based quantification. Streptomycin, tetracycline, vancomycin, and gentamicin was also evaluated against *B. subtilis*, *E. coli*, and *Micrococcus luteus* via blood agar plate assays.

Blood and Bacterial Strain Tests

Four initial conditions determined the extent of bacterial hemolysis: two trials assessed the impact of wildtype *E. coli* at 24h and 48h time points respectively; the other two trials replicated test conditions with a different bacterial strain, wildtype *B. subtilis*. Each condition was evaluated using six 1.5mL Eppendorf tubes for a total of 24 tubes. 500µL of heparinized sheep blood and 200µL of varying bacteria-broth concentrations were added to all tubes. Bacteria was diluted with Difco™ nutrient broth to create concentrations of 0%, 5.7%, 11.4%, 17.1%, 22.9%, and 28.6% in the tubes. The tubes were then vortexed and incubated in a water bath, where 12 tubes incubated for either 24h or 48h each. After incubation, contents were inverted five times to ensure homogenous mixing, and blood smears were made using 0.7µL from each tube. All tubes were then centrifuged at 1000 RCF for 10 minutes to separate the plasma for extraction, which was then added to a 96-well plate. 1:20 serial dilutions were performed for each plasma extract to avoid signal saturation from the plate reader. Following this, an end-point spectral scan from 300nm to 700nm was performed to determine absorbance values.

Introduction of Antibiotic Treatment

Results from tests determined that the 5.7% *E. coli* and 22.9% *B. subtilis* concentrations had the highest absorbance values of 4.05 AU and 3.82 AU

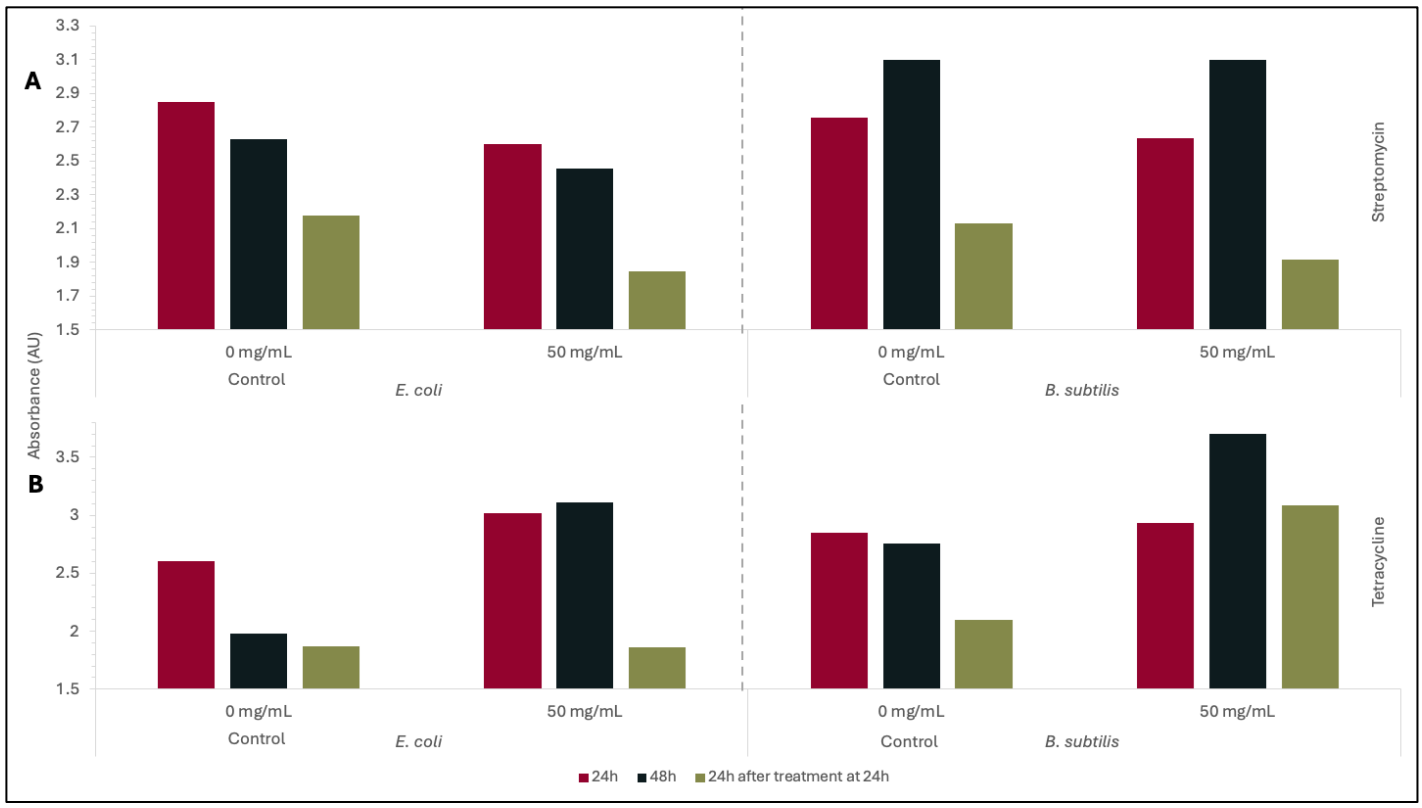

Figure 1. Absorbance values at 0 mg/mL and 50 mg/mL of antibiotic treatment, measured at 24h, 48h, and 24h after treatment at 24h. A) displays values of tubes treated with streptomycin, which showed a decrease in absorbance with increased drug concentration. B) displays values of tubes treated with tetracycline, which showed an increase in absorbance with increased drug concentration.

respectively at 24h. As a result, these two concentrations were standardized for the second round of tests, which introduced streptomycin and tetracycline. *E. coli* and *B. subtilis* broths were standardized to just above 0.1 AU to account for the aging blood. Both antibiotics were used to create a stock concentration of 50mg/mL, using DMSO as a solvent for tetracycline and dH_2O for streptomycin. Further dilutions of both stock solutions used dH_2O. There were 12 conditions tested: four conditions tested a bacterial strain (*B. subtilis* or *E. coli*) incubated with an antibiotic (streptomycin or tetracycline) for 24h, another four conditions replicated this set-up but instead incubated for 48h, and the final four conditions replicated the set-up, but antibiotics were added after 24h of bacteria-blood incubation to simulate an *in vivo* infection. For this latter group, measurements were taken after an additional 24h of incubation with the antibiotic (Figure 1).

Group 1: Inceptive Antibiotic Treatment

Each Eppendorf tube with *E. coli* (5.7%) contained 500µL of blood, 80µL of bacteria, 120µL of broth, and 50µL of antibiotic, testing six different concentrations: 0, 3.125, 6.25, 12.5, 25, and 50mg/mL. Each tube with *B. subtilis* (22.9%) contained 500µL of blood, 160µL of bacteria, 40µL of broth, and 50µL of antibiotic, resulting in six different concentrations with the same 2-fold difference as the *E. coli* tubes.

Serial dilutions were used to create varying concentrations. Blood, bacteria, and antibiotics were micropipetted into the tubes, followed by incubation for 24h and 48h. After incubation, tubes were

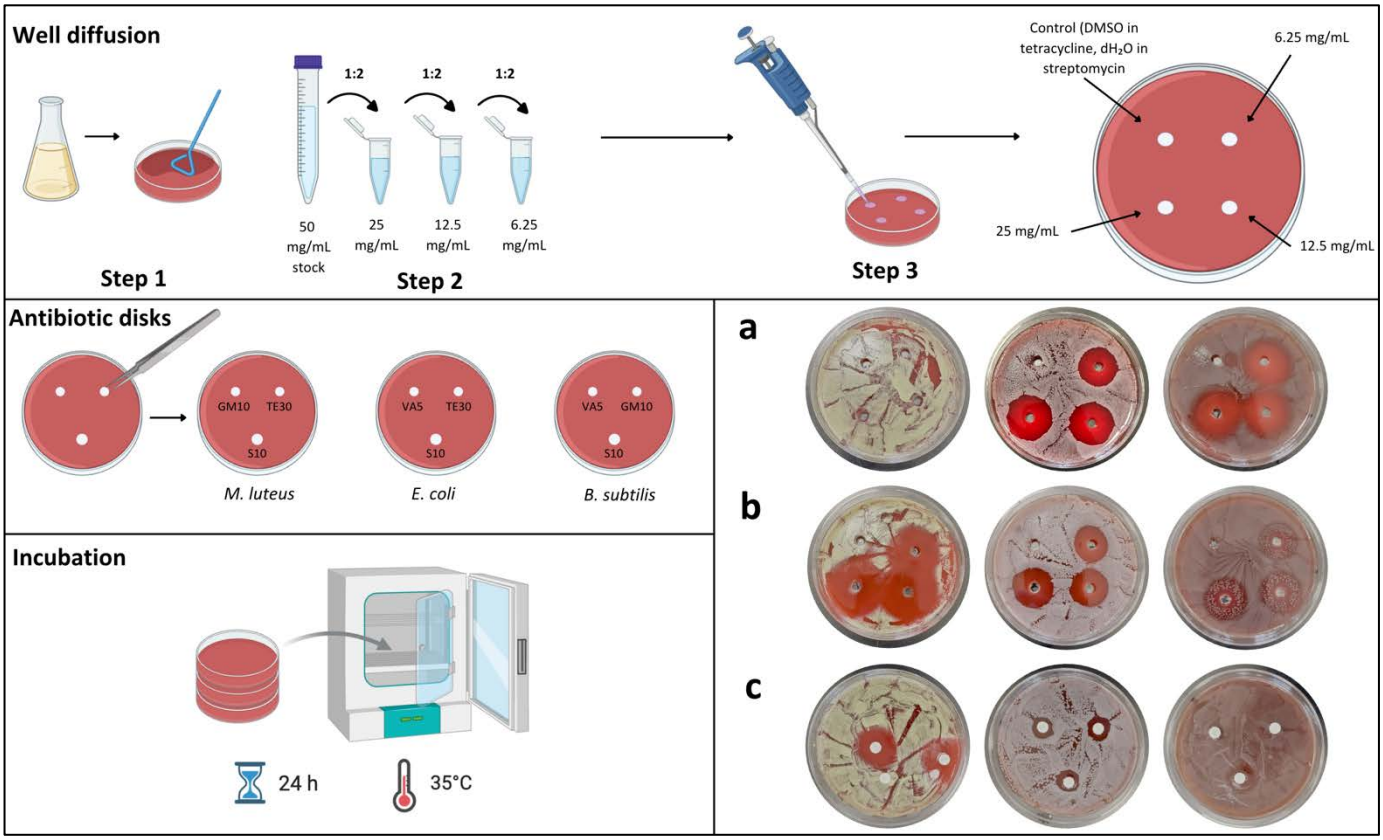

Figure 2. Setup of blood agar plates. Antibiotics were added using disks and the well diffusion method, where holes were created using a straw. A) shows plates treated with streptomycin. B) shows plates treated with tetracycline. C) shows plates treated with various antibiotic disks. Plates, from the leftmost to rightmost column, show growth of *M. luteus*, *B. subtilis*, and *E. coli*.

inverted five times and blood smears were made with 0.7µL of blood. Tubes were centrifuged for 10 minutes at 1000 RCF. Plasma extracts were highly saturated, so a 2.5% concentration was created in the well plate by aliquoting 5µL of plasma and 95µL of dH_2O into a well, then performing a 2-fold dilution.

Group 2: Delayed Antibiotic Treatment

The tubes in Group 2 were prepared identically to Group 1, however the 50µL of antibiotic was added after 24h of incubation. Plasma extraction and well plate procedures occurred in the same manner as Group 1.

Blood Agar Tests (Well Diffusion)

150µL of *E. coli*, *B. subtilis*, and *M. luteus* were spread evenly on the surface of Fisher Scientific blood agar plates via a cell spreader. Holes with a diameter of 0.6cm were made in the plate quadrants using a straw, and 20µL of three antibiotic concentrations (6.25, 12.5, and 25mg/mL) was micropipetted into each well, with each plate testing either streptomycin or tetracycline. DMSO was used as the control well in tetracycline treatments, while dH_2O was used in streptomycin treatments (Figure 2a, 2b).

Blood Agar Tests (Antibiotic Disks)

Bacteria-broth was spread across the surface of blood agar plates and three antibiotic disks were placed in each plate. Disks were chosen based on hypothesized effectiveness against the bacteria, depending on gram-positive or gram-negative natures. Streptomycin and tetracycline (broad-spectrum) as well as vancomycin (effective against gram-positive bacteria) were tested on *E. coli* (gram-

negative). Tetracycline, streptomycin, and gentamicin (effective against gram-negative bacteria) were tested on *M. luteus* (gram-positive). Vancomycin, streptomycin, and gentamicin were tested on *B. subtilis* (gram-positive) (Figure 2c).

Results

Regardless of incubation period, streptomycin tubes displayed a decrease in absorbance values compared to the control, while tetracycline tubes saw increased absorbance values with increased drug concentration. One potential reason for this is that tetracycline itself can induce hemolysis.[5] Additionally, increased hemolysis in *E. coli* specifically could be a result of tetracycline being partially ineffective against it, as displayed in the corresponding blood agar plate.

In blood agar plates, *M. luteus* displayed gamma hemolysis, as the agar surrounding its growth remained unchanged after incubation. The agar around *E. coli* and *B. subtilis* growth was transparent, showing beta hemolysis. Zones of inhibition (ZOI) increased in diameter in accordance with increasing drug concentration in all agar plates, regardless of the condition. The one exception to this was streptomycin and *M. luteus*, which displayed no ZOIs. Additionally, the *E. coli* and tetracycline plate still had colonies growing on the ZOI, indicating that the treatment was partially ineffective. The results of the antibiotic disks aligned with hypotheses, albeit with some differences; tetracycline was not effective in *E. coli*, gentamicin was effective in *B. subtilis* and *M. luteus*, and streptomycin was not effective in *M. luteus*. This contradicted well diffusion results, which could be attributed to differing properties of antibiotics and bacteria in various growth mediums.[6]

This study showed that hemolysis could be mitigated with streptomycin treatment, while tetracycline potentially contributed to hemolysis. Future studies may further explore the properties of tetracycline to explore the effect of bacteria and antibiotics on isolated cells.

References

1. Müller, D. J. & Engel, A. Chapter 13 - Conformations, Flexibility, and Interactions Observed on Individual Membrane Proteins by Atomic Force Microscopy. in *Methods in Cell Biology* (eds. Jena, B. P. & Heinrich Hörber, J. K.) vol. 68 257–299 (Academic Press, 2002).
2. Skals, M., Jorgensen, N. R., Leipziger, J. & Praetorius, H. A. α-Hemolysin from Escherichia coli uses endogenous amplification through P2X receptor activation to induce hemolysis. *Proceedings of the National Academy of Sciences* **106**, 4030–4035 (2009).
3. Liu, J., Fang, C., Jiang, Y. & Yan, R. Characterization of a Hemolysin Gene ytjA from Bacillus subtilis. *Curr Microbiol* **58**, 642–647 (2009).
4. Akinjogunla, O. J. *et al.* Antibiotic Resistance Profile, Multidrug-, Extensively drug-, and Pandrug-Resistant Bacterial Isolates: Hemagglutination and Hemolytic Activities against Human Erythrocytes. *Proc. Natl. Acad. Sci., India, Sect. B Biol. Sci.* **95**, 207–222 (2025).
5. Mazza, J. J. & Kryda, M. D. Tetracycline-induced hemolytic anemia. *Journal of the American Academy of Dermatology* **2**, 506–508 (1980).
6. Rose, R. *et al.* Comparison of the properties of Bacillus subtilis spores made in liquid or on agar plates. *J Appl Microbiol* **103**, 691–699 (2007).

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labprac|methods BRIEF COMMUNICATION

Examining Bacterial Resistance in *Escherichia coli* Through Antibiotic Treatment

Soleil Goodwin and Julie Mesha

This study compared the efficacy of two classes of antibiotic drugs against *Escherichia coli* after treatment with a single dose versus the equivalent dose administered as multiple smaller doses. After determining the minimum inhibitory concentration (MIC) for the single dose of each drug, the remaining bacteria after treatment were cultured on agar plates. Three wells for gradient diffusion treatment were created in the agar with high and low concentrations of antibiotic and a control well of the drug diluent dimethyl sulfoxide. Zones of inhibition were measured to determine antibiotic resistance in the bacteria. This methodology was repeated for the interval treatment to determine if antibiotic resistance varied with the method of treatment.

Multidrug bacterial resistance continues to evolve and become a barrier in healthcare, as current treatments often fail to address drug-resistant strains in patients. Thus, bacterial infections that have had effective treatments are seeing a rise in fatalities[1]. This is evident in tetracycline, with its use declining due to resistance thus rendering it ineffective. From treating wastewater to lung disease, azithromycin also faces bacterial resistance[2,3]. While most research focuses on the development of new drugs, little research focuses on altering the dose and frequency of drug administration in current drugs. Given tetracycline is bacteriostatic, and azithromycin is bactericidal, it was hypothesized that resistance may vary depending on both drug class and dosing size. By testing multiple classes of antibiotics and administration strategies, this study aims to identify the factors that lead to resistance during antibiotic treatment.

Escherichia. coli and Antibiotic Preparation

A wild-type strain of *E. coli* was grown for 24 hours in Difco™ Nutrient Broth in a 35°C water bath and subsequently standardized to an absorbance of 0.08-0.1 AU. Concentrations of azithromycin and tetracycline (Millipore Sigma) were chosen based on a range of MIC values identified by previous studies[4,5]. Stock concentrations of 0.256 mg/mL for azithromycin and 0.512 mg/mL for tetracycline were prepared in dimethyl sulfoxide (DMSO). All MIC concentrations from previous literature were less than these values and fell within the 12-well range of two-fold serial dilutions.

MIC Determination and Culture

In a 96-well plate, 50µL of sterile growth media was added to columns 2 through 12. 100µL of stock azithromycin was put in rows A-C of column 1 and 100µL of stock tetracycline was placed in rows D-F of the same column. Two-fold serial dilutions of 50. ML was performed across each row. *E. coli* (50 µL) was then added to each for a final volume of 100µL. Positive controls (50µL media and 50µL *E. coli*), and negative controls (100µL media) were in row H. The plate was incubated for 24 hours at 35°C and subsequently placed in a Biotek Synergy HTX plate reader to document endpoint absorbance values at 600 nm wavelength. Each row was examined to identify the minimum inhibitory concentration of either drug. Inhibited growth was determined based the greatest increase in absorbance values. The MIC well was cultured on agar, treated through well

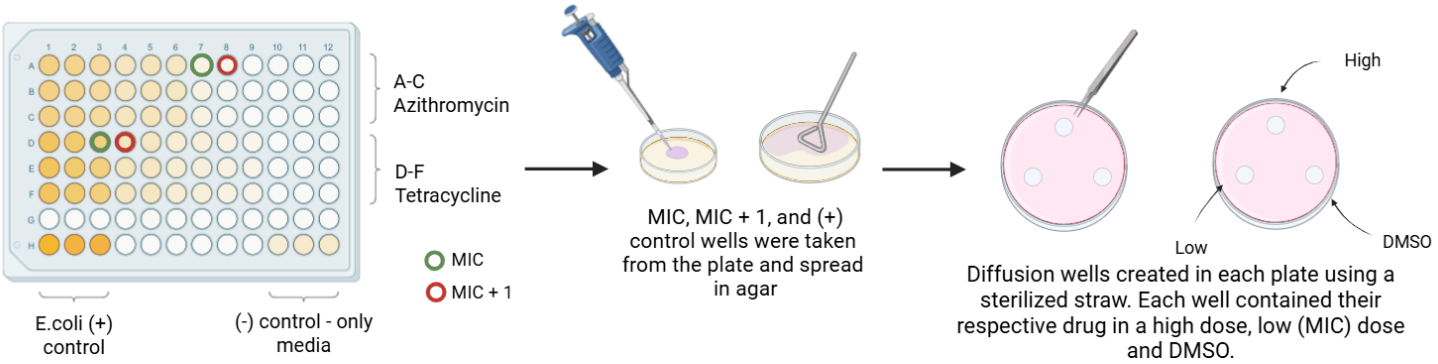

Figure. 1 | A schematic overview of wells chosen and agar plate preparation. The green wells are the MIC—determined by the greatest increase in absorbance value to the next well. The red wells are MIC+1, containing half the concentration of the MIC. Positive controls containing media and *E. coli* were in row H. 100µL from MIC, MIC+1, and positive control wells were pipetted to a Difco™ agar plate and spread with a sterile cell spreader. Diffusion wells were created in the agar using sterilized straws and forceps. Each plate contained a high dose, low dose, and DMSO well.

diffusion and incubated at 35°C for 24 hours (Figure 1). Plates contained a 'high dose' treatment, which was the maximum tested dose and expected to inhibit growth, a 'low dose', which was the MIC dose, and control DMSO, that should not affect growth. For azithromycin, the high dose was 128 mg/L, and the low dose was 2 mg/L. For tetracycline the high dose was 256 mg/L, and the low dose was 32 mg/L. Each treatment well contained 20µL of solution. The same protocol was applied to the well to the right of the MIC (MIC+1) in case there was no growth from the MIC wells. This experiment was done to compare the presence of resistant and drug-sensitive strains of bacteria upon secondary treatment.

Multi-dose Treatment and Culture

This experiment investigated the effects of administering multiple smaller doses in intervals on resistant-strain growth and MIC concentration. The same drug doses from the two-fold serial dilution used previously were prepared in a separate 96-well drug plate. The 50µL drug dose was added to the *E. coli* in 10µL increments at 2-hour intervals (hours 0-8). The treatment plate contained 50µL *E. coli* and 40µL sterile growth media, with the remaining 10µL being the initial drug dose. To maintain a consistent volume of 100µL, before each subsequent dose, 10µL was removed from each well in the treatment plate. Rows A-C were treated with azithromycin and rows D-F with tetracycline. To confirm that the interval dosing followed the expected growth trend versus single dose treatment, the plate was incubated at 35°C in a plate reader between doses and for an additional 18 hours after the final dose, totally 26 hours. The absorbance was documented through a kinetic assay, with 25 reads recorded per hour with continuous orbital shaking. The controls were the same as previous, with an additional positive control representing the multi-dose procedure This would confirm if the process of removal and addition affected growth compared to the other positive control. MIC values were determined from endpoint absorbance readings. Similarly to the single dose experiment, MIC and MIC+1 wells were cultured on agar plates and treated through gradient diffusion. Agar plates were again incubated at 35°C for 24 hours, and zones of inhibition were determined.

Single- and Multi-dose Treatment and Culture

With single-dose MICs determined to be within the serial dilution range and multi-dose effectiveness confirmed, the experiments were repeated at the same time using a single batch of standardized *E. coli*. Two 96-well plates were used: one for single-dose and

one for multi-dose treatment. The single-dose plate followed the same process as the initial experiment, and was repeated to ensure *E. coli* underwent the same conditions and came from the same batch as the multi-dose. The multi-dose plate followed the same process as its initial treatment, but was incubated in an oven, as only the endpoint plate reading was necessary. Plates were incubated for 24 hours, with multidose receiving doses every 2 hours from hours 0-8.

Results

The MIC revealed the relationship between modality and concentration of each drug needed to effectively inhibit bacterial growth. The azithromycin MIC was 2 mg/L whereas the MIC for tetracycline was 32 mg/L, meaning to kill relatively the same number of bacteria, a higher concentration of tetracycline is needed than azithromycin. Agar plate Inhibition zone diameters were compared across treatments using t-tests ($p<0.05$). Azithromycin demonstrated significantly higher efficacy in killing resistant bacteria than tetracycline (Figure 2). High and low doses for both the MIC and MIC+1 showed distinct zones of inhibition. Tetracycline's MIC and MIC+1 plates showed no zones of inhibition for any concentrations of treatment. The control well agar did show inhibition when treated with tetracycline. This revealed tetracycline's inefficacy against resistant strains at both tested dose concentrations.

Administering the dose in intervals did not significantly change this outcome, and neither did it change the MIC value for each drug. The experiment confirmed that treatment would be just as effective when administered in multiple doses. The growth curves constructed from the kinetic assay data aligned with the modality of each treatment, with azithromycin killing the bacteria and tetracycline keeping the bacterial population static. This result was confirmed on agar plates, as resistant bacteria continued to grow even after treatment with tetracycline, but not with azithromycin. When running the interval and single dosing concurrently it was clear that the growth in agar was not significantly different. The drug modalities showed a significant

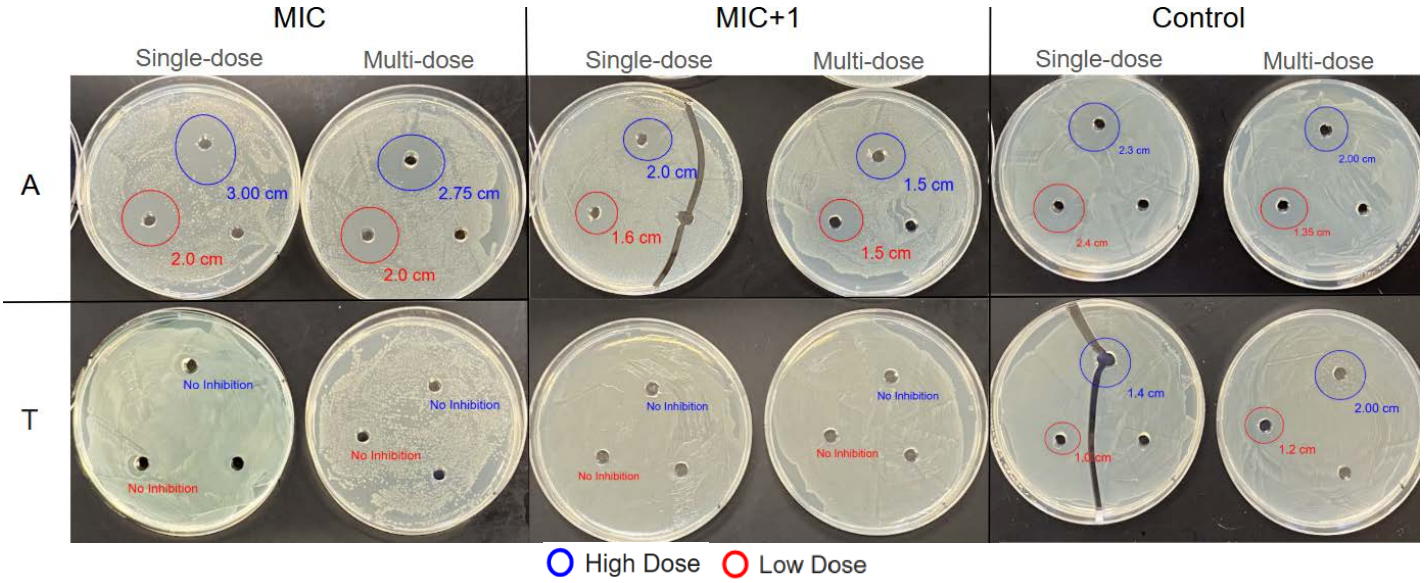

Fig. 2 | Agar Plate comparison of azithromycin-(A) and tetracycline-treated (T) MIC, MIC+1 and control well *E. coli*. Azithromycin treatment showed zones of inhibition across samples from all tested wells. Tetracycline showed no inhibition in previously treated *E. coli*, as inhibition zones were only present in untreated control. This showed a significant different in inhibition between A and T for MIC and MIC+1, with insignificant differences in control growth. No significant differences in growth occurred between single- and multi-dose for any condition.

difference, rather than the type of treatment administration. Tetracycline showed no efficacy against resistant strains, while azithromycin–high and low doses–did. The bactericidal action of azithromycin likely sustained inhibition of resistant strains, while tetracycline's failure to inhibit indicates growth of resistant bacteria after the static effect of its treatments.

Conclusion

Utilizing specific classes and concentrations in antibiotic treatment is crucial to effectively kill both drug-sensitive and drug-resistant bacteria. Though MICs from both drugs inhibited growth in the 96-well plates, their bacteriostatic modality yielded significantly more resistance than bactericidal when retreated. Future studies may investigate the efficacy of doses between the high and MIC dose that can optimize efficacy without resistant strain growth. Observing the behavior of resistant bacteria allows understanding of optimized treatment.

References

1. Rudra, P., Hurst-Hess, K., Lappierre, P. & Ghosh, P. High Levels of Intrinsic Tetracycline Resistance in Mycobacterium abscessus Are Conferred by a Tetracycline-Modifying Monooxygenase. *Antimicrob Agents Chemother* **62**, e00119-18 (2018).
2. Abotsi, R. E. *et al.* The impact of long-term azithromycin on antibiotic resistance in HIV-associated chronic lung disease. *ERJ Open Res* **8**, 00491–02021 (2021).
3. Tabrizi, A. M. A., Kakhki, S., Kakhki, S., Foroughi, M. & Azqhandi, M. H. A. Azithromycin resistance genes in *Escherichia coli* isolated from wastewater: Characterization and modeling-based evaluation of factors affecting the prevalence. *Process Safety and Environmental Protection* **168**, 32–41 (2022).
4. Chalmers, G., Kozak, G. K., Hillyer, E., Reid-Smith, R. J. & Boerlin, P. Low minimum inhibitory concentrations associated with the tetracycline-resistance gene tet(C) in Escherichia coli. *Can J Vet Res* **74**, 145–148 (2010).
5. Uwamino, Y. *et al.* Minimum inhibitory concentrations of azithromycin in clinical isolates of Mycobacterium avium complex in Japan. *Microbiology Spectrum* **12**, e00218-24 (2024).4

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labprac|methods BRIEF COMMUNICATION

Evaluating Moringa Oleifera as a Low-Cost Alternative for Water Purification in Resource-Limited Communities

Ahad Syed and Cassie Ephrem

Commercial water purification methods, such as tablets and UV disinfection, are often costly and impractical for underdeveloped regions. This study explores *Moringa oleifera* seed powder as a low-cost alternative due to its antimicrobial properties. Contaminated water was treated with either Moringa seed powder, two types of purification tablets, or UV-C light exposure. Effectiveness was measured by bacterial colony growth on agar plates. The tablets were found to inhibit most of the tested bacteria concentrations, supporting their effectiveness as commercial water treatments. UV-C sterilization was found to be less effective in our study and may have led to the reduced efficacy of the tablets. Moringa showed dose-dependent bacterial inhibition, highlighting its potential for applications in low-resource communities. Further research is needed to assess its long-term stability and real-world applications.

Introduction

Access to potable water is a fundamental human right, however, millions of people worldwide are still facing the daily challenge of obtaining clean water for consumption.[1] The global water crisis is particularly severe in developing countries found in Asia and Africa, where inadequate infrastructure and economic constraints hinder access to reliable purification methods. Contaminated water remains a leading cause of disease and mortality, with pathogens such as *Escherichia coli* and *Bacillus subtilis* posing serious health risk.[2] While commercial purification methods are used, such as chemical treatment, tablets and ultraviolet (UV) disinfection, they are often costly or impractical for the population to utilize in these underdeveloped areas.

Research into affordable and sustainable water purification solutions has grown as a response to this issue. Natural and low-cost alternatives have the potential to bridge the gap in clean water accessibility, which will offer practical solutions for communities that lack many resources. *Moringa oleifera*, a plant native to many countries in South America, South Asia, and Africa has gained attention for being a natural and low-cost alternative for treating contaminated water due to its microbial and coagulative properties.[3]

In this study, the efficacy of Moringa seed powder as a natural water purification method was investigated and evaluated by comparing its bactericidal capabilities against two types of commercial purification tablets and UV disinfection. By determining an optimal concentration of Moringa treatment that produces comparable results to conventional methods, this research aims to contribute to the growing body of sustainable purification solutions.

Methods

Simulating Contaminated Water

To simulate contaminated water, standardized cultures of wildtype *E. coli* and *B. subtilis* were used at varying concentrations. 10 mL of sterile water was added to a sterile 15 mL Falcon tube and proceeded to perform serial dilutions to achieve final bacterial concentrations of 2.5%, 0.25%, 0.025%, and 0.0025%. Contaminated water solutions were made fresh as

needed, and all bacterial concentrations were streaked to serve as controls for the corresponding treated samples.

Aqua-Tab Treatment

The AquaTab (AT) treatment was prepared by dissolving one tablet in 10 mL of sterile distilled water within a 15 mL Falcon tube, followed by vortexing until the tablet was completely dissolved. 1 mL of each contaminated water concentration was transferred to sterile 1.5 mL Eppendorf tubes, followed by 10 µL of the AT treatment. The treated solutions were placed on a rotator at 40rpm for 10 minutes to ensure proper mixing and were left undisturbed for 30 minutes as per the instructions on the packaging.

Potable Aqua Treatment

The Potable Aqua (PA) Treatment was prepared by dissolving two tablets into 10 mL of sterile distilled water within a 15 mL Falcon tube. The treatment was left to sit for 5 minutes before being vortexed for 30 seconds to ensure proper dissolving. 1 mL of each of the contaminated water concentrations was added to 1.5 mL Eppendorf tubes and treated using 10 µL of the PA treatment. Post-treatment, all solutions were left undisturbed for 30 minutes as per the instructions on the packaging.

UV-C sterilization

UV light is unable to penetrate plastic barriers, which would compromise the effectiveness of the UV treatment. Since our contaminated water samples were initially prepared in plastic Falcon tubes, we adapted our methodology by using sterile glass tubes to ensure proper UV exposure. Contaminated water samples of all concentrations were placed into a UV sanitation box and were treated for 10 or 20 minutes. Some of the contaminated water samples were treated with UV alone, while others were pre-treated with a quarter-dose of either the AT or PA tablet treatment and then received UV exposure to assess the combined effect of these disinfection methods.

Moringa Seed Powder Treatment

The Moringa seed powder treatment was prepared by weighing out 0.1 g of Moringa seed powder (Obtained from Trade Technocrats LTD), on a weighing scale. The powder was added to 10 mL of sterile, distilled water in a 15 mL Falcon tube and mixed by inversion seven times before being vortexed for 30 seconds to create a homogeneous mixture. Different treatment doses (32, 64, 128, and 256 µL) of the Moringa

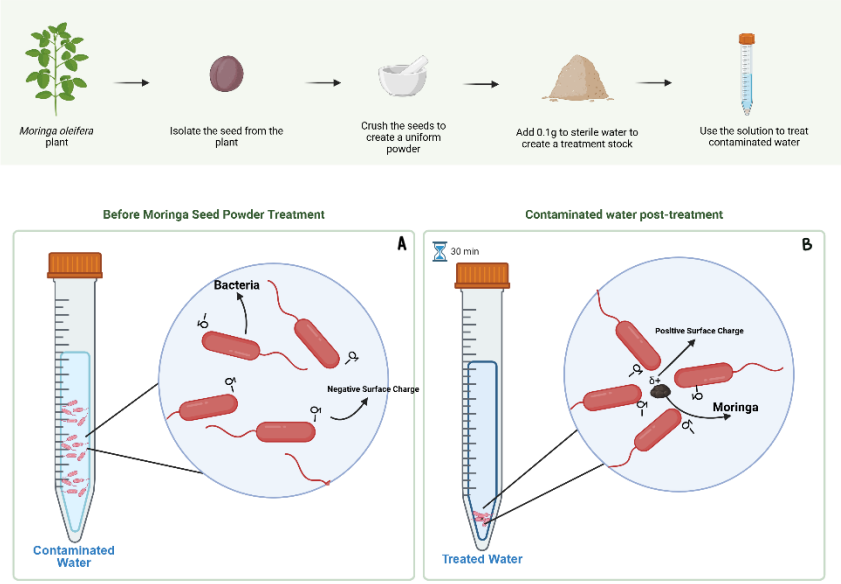

Figure 1. Moringa oleifera seed powder has the potential to act as a natural water treatment agent. The top section outlines the general preparation of the Moringa treatment stock. In panel A, bacterial cells (such as E. coli and B. subtilis) are dispersed throughout the water with an overall negative surface charge and remain suspended. In panel B, Moringa seed powder introduces positively charged proteins, which interact electrostatically with the negatively charged bacterial surfaces allowing bacterial cells and other suspended particles to aggregate into larger flocs, a process known as flocculation. These flocs settle at the bottom of the tube, leaving clearer water

above. This flocculation mechanism is key to Moringa's effectiveness as a natural coagulant, aiding in bacterial removal by sedimentation rather than direct bactericidal action.[4]

treatment was added to 1 mL of each contaminated water concentration in 1.5 mL Eppendorf tubes. The treated solutions were then left undisturbed for 30 minutes for flocculation to occur (Figure 1).

Plating Procedure

All the treated solutions were streaked onto DIFCO agar plates following a predesigned template to maintain streaking pattern consistency. Plates were incubated and subjected to colony count assessments. Efficacy of each treatment was determined by comparison to control plates. For the Moringa treatment, only the top third of the Eppendorf tube solution was used while streaking to avoid disrupting the settled particles at the bottom.

Results

The efficacy of various treatments in inhibiting bacterial growth was tested and evaluated for *E. coli* and *B. subtilis* at different concentrations (Figure 2). PA and AT were effective across all bacterial concentrations, with PA completely inhibiting bacterial growth. Furthermore, AT showed complete inhibition at all *B. subtilis* and *E. coli* concentrations except for 2.5% *E. coli*. The UV treatment alone at both 10 and 20 minutes demonstrated mixed efficacy, with only partial inhibition at the lower *E. coli* concentrations and did not affect 2.5% *E. coli*. Similarly, when treating *B. subtilis* with UV exposure at 10 or 20 minutes, none of the exposure times were found to be effective in our system.

Moringa seed powder exhibited a dose-dependent effect. At the lower dosages (32 and 64 µL), no inhibition was observed against either bacterium, no matter the concentration of bacteria being treated. However, at the higher dosages (128 and 256µL), and lower bacteria concentrations (0.025% and 0.0025%) growth was completely inhibited for both strains.

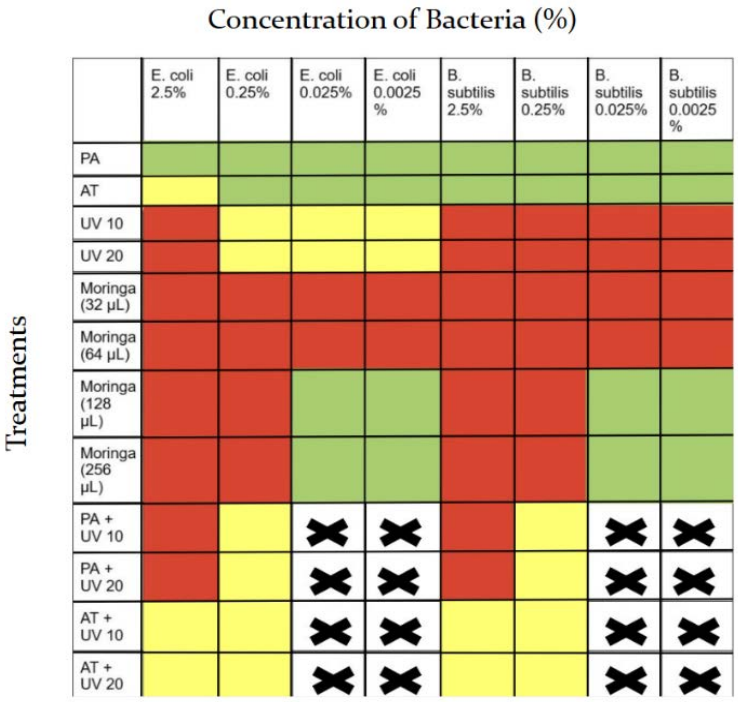

Figure 2. Chart displaying the effects of various treatments on bacterial growth inhibition for *E. coli* and *B. subtilis* at different concentrations. The results are summarized in the chart, where green indicates complete inhibition, yellow represents partial inhibition, and red signifies no inhibition.

The combinations of PA or AT tablets with the UV treatments at 10 or 20 minutes were either ineffective or only partially effective. The low bacteria concentrations (0.025% and 0.0025%) were untested in the combination treatment, which is marked with a black 'X' in Figure 2. PA + UV was found to have no effect on the 2.5% bacteria concentration for both bacterial strains. The remaining combination treatments showed partial effectiveness for all tested bacterial concentrations. When combined with both tablet treatments to test the synergistic effect, the results showed that rather than inhibiting bacterial growth at all, UV sterilization may have reduced the

efficacy of each tablet and led to more growth of bacteria.[5] Overall, both tablet treatments (PA and AT) and the higher doses (128 and 256µL) of Moringa treatment were most effective in bacterial inhibition in our study. The lower doses of Moringa, the UV treatments alone, and the UV + tablet treatments were found to be less effective or ineffective in our study.

Conclusion

Our study indicates that Moringa seed powder can serve as a promising low-cost alternative for existing water purification methods, especially in low-resource communities. Future studies could examine the effect of storage on Moringa-treated water, checking to see if it is still effective or if bacterial regrowth occurs during storage. Similarly, field trials can be conducted in communities that rely on contaminated water to assess real-world effectiveness and feasibility. In conclusion, our results suggest that natural and accessible alternatives like Moringa seed powder may offer viable supplemental solutions to current water treatment methods and could provide a more affordable solution in water-scarce communities.

References

1. Dandesa, Barecha, Desalegn Abdissa Akuma, and Esayas Alemayehu. 2023. “Water Purification Improvement Using Moringa Oleifera Seed Extract Pastes for Coagulation Follow Scoria Filtration.” *Heliyon* 9 (7): e17420.

2. Desta, Wendesen Mekonin, and Million Ebba Bote. 2021. “Wastewater Treatment Using a Natural Coagulant (Moringa Oleifera Seeds): Optimization through Response Surface Methodology.” *Heliyon* 7 (11): e08451.

3. Kwabena Ntibrey, Richard Agbo, Francis Atta Kuranchie, and Samuel Fosu Gyasi. 2020. “Antimicrobial and Coagulation Potential of *Moringa Oleifera* Seed Powder Coupled with Sand Filtration for Treatment of Bath Wastewater from Public Senior High Schools in Ghana.” *Heliyon* 6 (8): e04627.

4. Scientific Image and Illustration Software | BioRender. Accessed April 2, 2025.

5. Mitxelena-Iribarren, Oihane, Beñat Mondragon, Eva Pérez-Lorenzo, Cristian Smerdou, Francisco Guillen-Grima, J. Enrique Sierra-Garcia, Fernando Rodriguez-Merino, and Sergio Arana. 2022. “Evaluation of the Degradation of Materials by Exposure to Germicide UV-C Light through Colorimetry, Tensile Strength and Surface Microstructure Analyses.” Materials Today Communications 31 (June):103690.

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