Comparative Antimicrobial Activity of Natural Honeys and Antibiotics Against *Escherichia coli* and *Staphylococcus aureus* An in vitro study.

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Abstract

Background: Natural honey has long been used as a therapeutic agent due to its broad-spectrum antimicrobial properties. Natural honey means unprocessed and left in a natural state after harvest. Activity arises from factors such as acidic pH (3.5–4.2), high osmolarity, hydrogen peroxide production, and diverse phenolic compounds. Components inhibit over 60 species of Gram-positive and Gram-negative bacteria, supporting honey's potential as an alternative treatment amid increasing antibiotic resistance.

Objective: We evaluated the antibacterial effects of honey on *Staphylococcus aureus* and *Escherichia coli* by determining whether undiluted honey could induce measurable bacterial cell death.

Methods: Experiments were performed under standard biosafety conditions using nutrient agar and tryptic soy agar plates inoculated via streak-plate method. Honey samples were deposited into 5-mm agar wells, with penicillin and erythromycin serving as antibiotic comparators. Plates were incubated at 37 °C for 24–36 hours, and zones of inhibition were measured to quantify antimicrobial activity.

Results: All honey types demonstrated antimicrobial activity against *E. coli*, producing zones of inhibition ranging from 2.36 to 5.20 mm. Manuka honey (MH) and Linden Honey (LH) produced the greatest inhibition among honey-only treatments. In the penicillin assay, honey–penicillin combinations showed variable enhancement, with MH+P and LH+P exhibiting the largest increases relative to honey alone. In the erythromycin assay, honey treatments produced inhibition comparable to or greater than erythromycin alone, and honey–erythromycin combinations generally maintained or modestly improved activity. Blackburn College honey (honey harvested from bee hives on campus) from fall and spring harvests displayed consistent antibacterial effects across trials.

Conclusion: Honey demonstrated intrinsic antibacterial properties in combination with antibiotics. Positive results were seen in all trials with the inclusion of honey including trials without antibiotic inclusion.

Author Summary

A rising antibiotic resistance presents complications for public health moving forward if no alternative antibacterial methods are developed. The study aimed to investigate various honey's ability to combat clinically relevant species. Outcomes of the study showed a positive effect on *Escherichia coli* and *Staphylococcus aureus* with all four honeys used. MH, LH, and Blackburn Spring honey (BHS) performed within close ranges of each other with an increase in antibacterial effectiveness when paired with antibiotics. Blackburn Fall honey (BFE) had minor inhibitory effectiveness despite being paired with antibiotics

Introduction

Honey has been used therapeutically for millennia, owing to its diverse biological, antimicrobial, and wound-healing properties (Pătruică et al., 2022). As antibiotic resistance continues to rise globally, there is growing interest in honey as an affordable and broadly accessible alternative to conventional antimicrobial therapies (Ogwu and Izah, 2025). Honey is a chemically complex substance composed primarily of sugars and water, but it also contains a wide array of bioactive compounds, including phenolic acids, flavonoids, antioxidants, and enzymatically produced antimicrobial agents (Ogwu and Izah 2025). Components collectively contribute to honey's ability to inhibit microbial growth while supporting tissue repair and immune modulation.

The antimicrobial activity of honey has been attributed to several mechanisms. Phenolic compounds and flavonoids determine antioxidant capacity, which varies by floral source, geographic location, storage conditions, and season of harvest (Pătruică et al., 2022). Antioxidants reduce reactive oxygen species (ROS) and alleviate oxidative stress, contributing both to antimicrobial efficacy and pain reduction in wound (Lubis et al., 2021). ROS are highly reactive oxygen molecules that damage tissues as they try to heal. Oxidative stress is the inflammation in the body caused by ROS constantly trying to react and damaging tissues. Honey's naturally acidic pH (3.5-4.2) inhibits bacterial proliferation, while glucose oxidase–mediated production of hydrogen peroxide further enhances antimicrobial action by maintaining a hostile, oxidative environment (Iftikhar et al., 2022 ; McArdle et al., 2023) Certain honeys, such as Manuka honey, contain methylglyoxal (MGO), a compound known to disrupt microbial biofilms and inhibit bacterial growth more directly (Ogwu and Izah 2025). Biofilms are an aggregation of bacterial cells that make it harder to get antibiotics into the cell and stop the growth and reproduction.

Honey's high sugar content creates a strong osmotic gradient that dehydrates bacterial cells, leading to collapse and inhibition (Lubis et al., 2021). Various mechanisms allow honey to target a wide range of Gram-positive and Gram-negative bacteria, and bacteria exposed to honey rarely develop resistance (Pătruică et al., 2022; McArdle et al., 2023). Gram-positive bacteria have one thick peptidoglycan wall while Gram-negative bacteria have one thin peptidoglycan wall, but a thick lipid layer underneath that's hard to penetrate.

In parallel with its antimicrobial properties, honey is also recognized for its wound-healing capabilities. Honey downregulates pro-inflammatory cytokines, reduces protease activity (enzymes that break down proteins), improves oxygen release from hemoglobin (the oxygen carrying molecule), and promotes collagen synthesis (connective tissue formation). Honey also encourages lymphatic clearance of debris and provides a moist, non-adherent environment that facilitates painless dressing changes and tissue regeneration (Kucharzewski et al., 2025).

Given its broad-spectrum activity, low cost, and minimal side effects, honey has demonstrated potential to complement clinical treatments, including antibiotics, and may outperform conventional antiseptics in certain contexts such as oral care (McArdle et al., 2023). However, challenges such as allergen content, risk of contamination, and sugar load in diabetic patients must be considered (McArdle et al., 2023). Further research is needed to evaluate honey's efficacy against antibiotic-resistant strains, characterize its bioactive constituents, and understand how factors such as pH or tissue environment influence antimicrobial performance (Ogwu and Izah, 2025; Cho et al., 2024).

Together, honey properties are a promising natural therapeutic with significant potential for modern biomedical applications. Our study examines the antibacterial effects of multiple honey types on *Escherichia coli* and *Staphylococcus aureus, e*valuating their potential to induce bacterial cell death relative to standard antibiotic controls. We predicted that Manuka honey and MGO would be most effective against both bacterial strains. If the study shows that various honeys can combat clinically relevant strains seen in infections in the human population, larger studies can explore honeys implementation in clinical studies. Successful clinical trials can mean using honey as a viable treatment option allowing researchers more time to develop stronger antibiotics.

Methods

Laboratory Setting

All experimental procedures were conducted in the Mahan Science Building at Blackburn College, Carlinville, IL. Laboratory work was performed under the supervision and guidance of faculty from the Biology and Chemistry Department. All procedures adhered to standard biosafety protocols.

Nutrient Agar Preparation

Nutrient agar from Carolina Biological Supply was prepared by dissolving 23g of nutrient agar powder in 1 L of deionized water. The solution was heated on a hot plate until fully dissolved, then sterilized in an autoclave at 121°C for 15 minutes. After cooling to 55-50°C, the agar was poured into sterile petri dishes under aseptic conditions. To minimize condensation, petri dishes were placed in a laminar flow hood with lids ajar until fully set. Once solidified, dishes were

sealed with parafilm, labeled, and stored at 4°C until use. Each preparation yielded approximately 30 petri dishes with dimensions of 10 x 90mm.

Tryptic Soya Agar Preparation

Tryptic Soya Agar from Carolina Biological Supply was prepared by dissolving 40g of powder in 1 L of deionized water, The solution was heated on a hot plate until fully dissolved, then sterilized in an autoclave at 121°C for 15 minutes. After cooling to 55-50°C, the agar was poured into sterile petri dishes under aseptic conditions. To minimize condensation, petri dishes were placed in a laminar flow hood with lids ajar until fully set. Once solidified, dishes were sealed with parafilm, labeled, and stored at 4°C until use. Each preparation yielded approximately 30 petri dishes with dimensions of 10 x 90mm.

Bacterial Strains and Culture Conditions

Two bacterial strains were used in this study: *Escherichia coli (E. coli)*, a facultative anaerobe and *Staphylococcus aureus (S. aureus)*. Cultures were maintained on nutrient or tryptic soya agar and incubated at 37°C for a minimum of 24-36 hours. Incubation conditions were aerobic with low humidity present. Negative control was *E. coli* streaked onto nutrient agar plates with no inhibiting factors. Positive control was *E. coli* streaked onto nutrient agar plates with the inclusion of a penicillin disc in the first five trials and an erythromycin disc in the following five trials. Inoculation method was streak plating: streak from edge of plate to middle, streak one half of remaining agar area, streak diagonally any remaining agar area.

S. aureus was prepared by using .1µL of rehydration medium dissolving the lyophilized bacteria in the tube. All contents of the tube were placed in the tube containing the rest of the rehydration medium, placed in a test tube rack, and placed in the incubator for 24 hours at 37°C to promote growth. Negative control was *S. aureus* streaked onto tryptic soya agar with no inhibiting factors. Positive control was S. *aureus* streaked onto tryptic soya agar plates with the inclusion of a penicillin disc in the first five trials and erythromycin disc in the next five trials. Inoculation method was streak plating: streak from edge of plate to middle, streak one half of remaining agar area, streak diagonally any remaining agar area.

<u>Agar Slant Preparation</u>

Tryptic Soya Agar from Carolina Biological Supply was prepared by dissolving 40g of powder in 1 L of deionized water. The solution was heated on a hot plate until fully dissolved, then sterilized in an autoclave at 121°C for 15 minutes. After cooling to approximately 50°C, the agar was poured into sterile test tubes about ⅓ full using a funnel, then set slanted in a rack to cool at 4°C overnight. Removed test tubes from refrigerator and transferred 100µL of rehydrated

medium onto slant surface. Test tube rack was placed in the incubator at 37°C and tilted at an angle for 24 hours. After 24 hours, test tube rack was moved into the fridge with lid on test tube sealed until ready for inoculation.

Biosafety Level and Strain Information

For preparation and use of *E. coli,* BSL-1 conditions were used. For preparation and use of *S. aureus,* BSL-2 conditions were used. Both bacterial strains were ordered through Carolina Biological Supply; Item numbers were 155065 and 155554A.

Honey Treatment Preparation

Honey was left undiluted and did not go through autoclave sterilization aiming to maintain the natural properties of honey. To incorporate into plates, honey was dripped from a probe into a well that was 5mm in diameter and 2mm long. A total of 30µL of honey was in each well and all trials had two wells present in the plate.

Disc Diffusion Protocol

Two antibiotic discs were used in trials (Penicillin and Erythromycin) measuring 6.35mm in diameter. One disc each, in conjunction with two wells of MH, LH, and both Blackburn College honey samples. Discs and honey wells were set to form a triangle on the plate approximately four inches apart from each other. Antibiotic discs were dispensed using a self-tamping disc dispenser and pushed into the top of the plate. Plates were incubated for 24-36 hours agar side up to minimize condensation dripping down onto bacteria. Measurements were recorded and then divided to obtain the average zone of inhibition. Each zone of inhibition was evaluated by measuring from edge of well to farthest area of bacterial inhibition represented by a clear circle on the petri plate (zone of inhibition), a variation of the Kirby-Bauer method (Hudzicki 2009).

pH Analysis

Statistical Analysis

Mean ± SD calculated using Microsoft Excel. Significance tested with two-tailed t-tests ($\alpha = 0.05$). No correction for multiple comparisons was applied due to exploratory study design.

Results

Table 1. Inhibition zones produced by antimicrobial treatments against *E. coli*

Treatment	Mean (mm)	SD	n	p-value vs Control
Control	0	0	5	—
Penicillin (P)	0	0	5	—
Manuka Honey (MH)	5.20	2.57	5	0.0025
Linden Honey (LH)	4.54	1.55	5	0.0002
Blackburn Honey – Fall (BHF)	3.54	1.13	5	0.0000
Blackburn Honey – Spring (BHS)	4.46	0.89	5	0.0000

MH + Penicillin (MHP)	3.52	.91	5	0.0031
LH + Penicillin (LHP)	2.86	.01	5	0.0002
BHF + Penicillin (BHFP)	3.36	.06	5	0.0000
BHS + Penicillin (BHSP)	3.86	0.91	5	0.0000

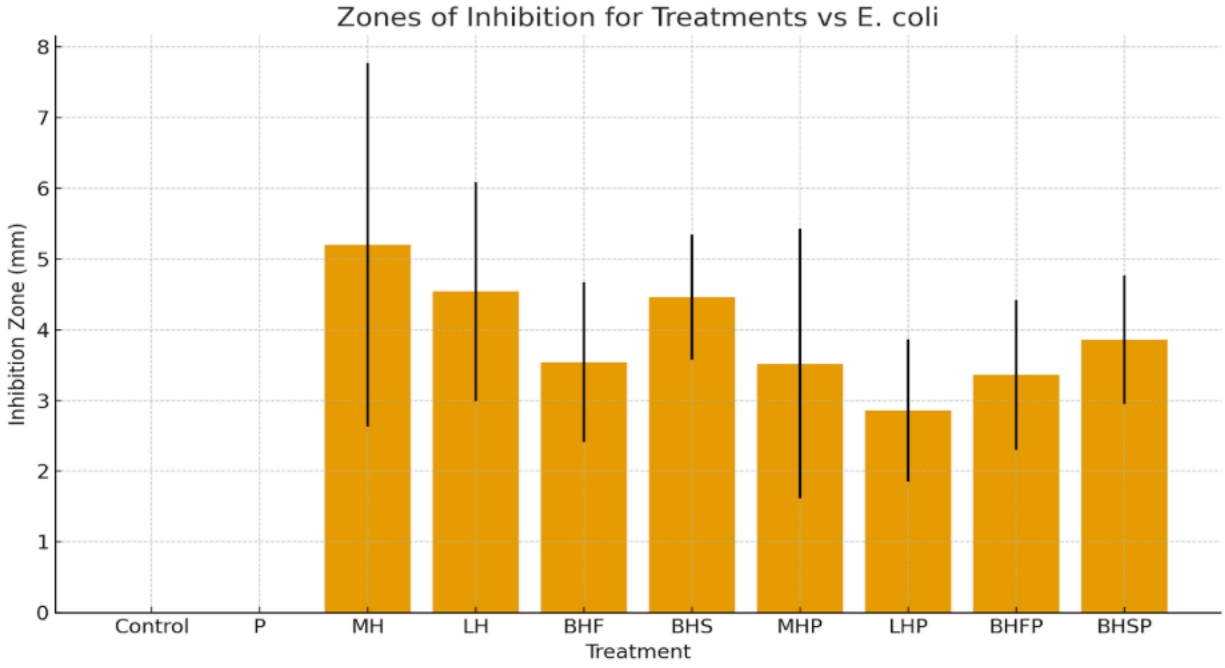

Fig. 1 Zones of inhibition including averages and standard deviation. Manuka honey produced the largest average inhibition zone (5.20 mm), followed by Linden honey and Spring Blackburn honey. Penicillin alone produced no inhibition, consistent with the known resistance of Gram-negative *E. coli* to β-lactam antibiotics. All honey treatments produced statistically significant inhibition relative to control ($p < 0.05$ for all comparisons). Combining honey with penicillin did not consistently increase inhibition compared with honey alone.

Table 2. Inhibition zones produced by antimicrobial treatments against *E. coli*

Treatment	Mean (mm)	SD	n	*p*-value vs Control
Control	0	0	5	—
Erythromycin (E)	3.2	0.45	5	0.0000
Manuka Honey (MH)	4	0	5	0.0000
Linden Honey (LH)	3.46	0.34	5	0.0000
Blackburn Honey – Fall (BHF)	2.36	0.50	5	0.0004
Blackburn Honey – Spring (BHS)	3.3	0.27	5	0.0000
MH + Erythromycin (MHE)	3.54	0.22	5	0.0000

LH + Erythromycin (LHE)	3.52	0.45	5	0.0000
BHF + Erythromycin (BHFE)	2.74	0.48	5	0.0002
BHS + Erythromycin (BHSE)	3.4	0.44	5	0.0000

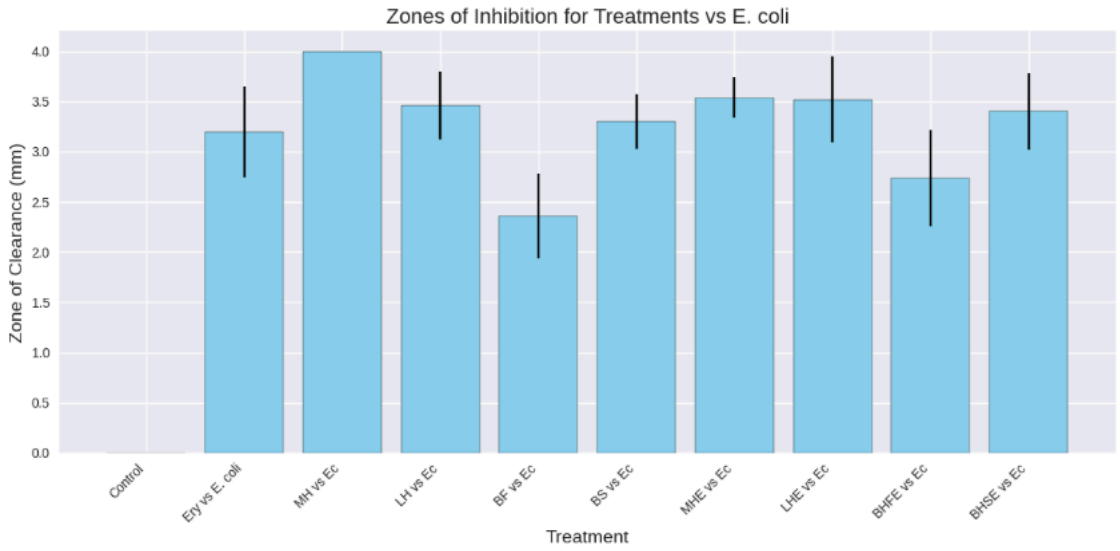

Fig. 2 Zones of inhibition including averages and standard deviation. Manuka Honey shows the highest inhibition (4.00 mm), followed closely by Manuka Honey+Erythromycin, Linden Honey+Erythromycin, and Blackburn Honey Spring+Erythromycin. Blackburn Honey Fall vs E. coli and Blackburn Honey Fall+Erythromycin had the lowest inhibition zones but were still statistically significant ($p < 0.05$). Erythromycin helped to combat bacteria more effectively compared to penicillin.

Discussion

Our study demonstrates that natural honeys, including locally collected varieties, exhibit significant antibacterial activity against *Escherichia coli*. Among the samples tested, Manuka

honey (MH) was the most effective at reducing bacterial growth, consistent with previous reports linking its potency to elevated methylglyoxal levels. Honeys collected from Blackburn College also produced moderate inhibition, supporting the view that honey's antimicrobial action is multifactorial, driven by osmotic stress, acidity, hydrogen peroxide release, and diverse phytochemicals. The absence of synergy between honey and penicillin suggests that honey does not facilitate β-lactam penetration into the periplasm of Gram-negative bacteria. This finding highlights the importance of bacterial cell envelope architecture in determining combination efficacy. Nevertheless, honey may still offer synergistic potential with other antibiotic classes, particularly those targeting protein synthesis or DNA replication, where oxidative stress and membrane disruption could enhance drug activity. Exploring these interactions remains an important avenue for future research. Clinically, honey's antimicrobial properties hold promise as a complementary therapy, particularly in topical applications such as wound care, where its multifactorial mechanisms may reduce the risk of resistance development. The natural origin and accessibility of honey further underscore its potential role in antimicrobial stewardship, offering an adjunct to conventional antibiotics in contexts where resistance limits treatment options. Future work should expand to multidrug-resistant pathogens, including MRSA and extended-spectrum β-lactamase (ESBL)-producing *E. coli*, and evaluate honey in combination with diverse antibiotic classes. Fractionation studies could identify specific phytochemicals responsible for synergy, while clinical trials will be necessary to establish efficacy and safety. Standardization of honey formulations will also be critical to ensure reproducibility and regulatory approval. Together, these findings reinforce honey's potential as a complementary antimicrobial agent in the era of rising antibiotic resistance. While synergy with penicillin was absent, the multifactorial nature of honey's activity suggests broader opportunities for combination therapy. Continued investigation into honey's mechanisms and clinical applications may position it as a valuable adjunct in global efforts to combat resistant infections.

Limitations

Our study has several limitations that should be considered when interpreting the findings. Only a single *E. coli* strain was evaluated, which restricts the generalizability of the results across other Gram-negative bacteria. The disc diffusion and well-diffusion methods used may underestimate antimicrobial activity, as viscous substances such as honey diffuse poorly through agar. To address poor diffusion in future studies, diluting honey to a lower viscosity may aid in better diffusion through the agar plate. Additionally, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were not determined, preventing quantitative assessment of antimicrobial potency. Limited chemical characterization of the honey samples was conducted including hydrogen peroxide content or methylglyoxal (MGO) levels, limiting insight into the specific components responsible for antibacterial effects.

Conclusion

Our study demonstrates that natural honeys exhibit significant antibacterial activity against *Escherichia coli*, whereas penicillin alone shows no effect due to intrinsic resistance. Among the honeys tested, Manuka honey displayed the strongest inhibitory effect, though locally sourced varieties also produced promising results. These findings support further investigation into honey as a complementary antimicrobial agent, particularly in the context of infections caused by antibiotic-resistant bacteria. Honey's multifactorial mechanisms—including osmotic stress, hydrogen peroxide production, and bioactive compounds—make it difficult for bacteria to develop resistance, underscoring its potential as a sustainable therapeutic option.

While our results highlight honey's efficacy in vitro, variability in composition across floral sources and regions necessitates broader comparative studies. Controlled clinical trials are essential to establish standardized dosing, formulation, and patient outcomes. Future research should also explore synergistic effects between honey and conventional antibiotics, as well as mechanistic insights into its antimicrobial pathways. Given honey's accessibility, affordability, and established use in wound care, oral health, and ulcer management, its integration into modern infection control strategies could be particularly impactful in low-resource and rural healthcare settings. In the face of escalating antibiotic resistance, honey represents a viable, cost-effective, and widely applicable antimicrobial that warrants serious consideration in global health efforts.

Supplementary Information

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