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Investigating Soil Microbiota for Antimicrobial Activity Against Safe Relatives of ESKAPE Pathogens

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Abstract

According to the CDC, there are more than 2.8 million antibiotic resistant infections occurring in the United States each year, and more than 35,000 people die as a result (CDC 2019). Furthermore, the CDC classifies a group of bacteria known as ESKAPE pathogens as six emerging antibiotic-resistant pathogens that are difficult to eradicate with current antibiotics. Our study aims to identify and characterize soil-derived microorganisms with the potential to produce antimicrobial compounds effective against safe relatives of ESKAPE pathogens, with the goal of translating these findings to combat their pathogenic counterparts. We hypothesize that bacteria identified from the soil will inhibit the growth of the following nosocomial associated safe relatives *Bacillus subtilis* for *E. faecium, Staphylococcus epidermidis* for *S. aureus, Escherichia coli* for *Klebsiella pneumoniae, Acinetobacter baylyi* for *A. baumannii, Pseudomonas putida* for *P. aeruginosa,* and *Enterobacter aerogenes* for *Enterobacter* species. To test our hypothesis, soil samples were collected from Fayetteville State University (FSU) campus and serially diluted onto LB agar plates. Sixty-three distinct colonies were isolated and screened against non-pathogenic ESKAPE safe relatives. Of the 63 Fayetteville State University soil isolates (FSIs) screened, 12 (19%) exhibited antimicrobial activity against at least one of the six ESKAPE safe relatives, with all 12 inhibiting *Acinetobacter baylyi* and only FSI 15 demonstrating broad-spectrum inhibition. Characterization assays revealed that 11 of the 12 isolates were Gram-negative, catalase-positive, and motile; the single Gram-positive isolate (FSI 4) was catalase-negative and non-motile. All isolates displayed resistance to penicillin, while most remained susceptible to tetracycline and ciprofloxacin. These findings support our hypothesis that soil-derived bacteria can produce putative antimicrobial compounds effective against non-pathogenic ESKAPE safe relatives. This study underscores the potential of soil microbiota on the campus of Fayetteville State University as a source of novel antimicrobial agents capable of inhibiting antibiotic resistant ESKAPE pathogens and warrant further investigation into their therapeutic potential.

Keywords: microbiology; antibiotic resistance; ESKAPE pathogens; soil.

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1. Introduction

In 1943, Dr. Selman Waksman a prominent microbiologist and biochemist, made groundbreaking discoveries in antibiotic development by exploring soil samples, leading to the identification of antibiotics like streptomycin which was a significant breakthrough in the treatment of tuberculosis. This was groundbreaking work that revolutionized the field of microbiology and pharmacology, particularly in the discovery of antibiotics derived from soil microorganisms (Opimakh 2023 and Baltz 2006). Dr. Waksman innovative techniques and insights into microbial competition allowed him to postulate that soil bacteria could secrete substances that inhibit other bacteria, thus opening a new avenue for antibiotic discovery (Ribeiro da Cunha et al., 2019; Hughes and Karlén, 2014). Dr. Wakman's platform for screening bacterial extracts set a precedent that dominated the domain of antibiotic research for decades, leading to significant breakthroughs during the 1940s and 1950s when many of the major antibiotic classes were identified (Hughes and Karlén, 2014; Lewis, 2013). However, as antibiotic resistance began to emerge, the novelty of discovering new antibiotics waned, leading to a significant decline in pharmaceutical interest in exploring soil-derived compounds. This indicated that although Waksman's discoveries were monumental, they also painted a complex picture of antibiotic development that continues to evolve today (Lewis, 2013). Currently, antibiotic discovery has stagnated, and antibiotic resistance continues to rise. Globally in 2019, this issue is estimated to have claimed 1.27 million lives by antibiotic-resistant infections. To better understand the ecological and physiological traits of bacteria that may influence their antimicrobial capabilities, it is important to consider their fundamental cellular structures. Bacteria can be broadly categorized as either Gram-positive or Gram-negative, each exhibiting unique characteristics that enable them to thrive in their respective environments. Gram-positive bacteria are characterized by their thick peptidoglycan cell walls, which provide robust protection against environmental threats (Wang et al., 2023; Hessle et al., 2000). In contrast, Gram-negative bacteria lack a substantial peptidoglycan layer but compensate with various virulence factors that enhance their adaptability (Wang et al., 2023; Tsuchido et al., 2019).

The rise of antibiotic-resistant bacteria is a pressing global health crisis attributed to several connected factors (overuse and misuse of antibiotics in human medicine, veterinary practices, and agricultural settings) that exacerbate effective antimicrobial treatment challenges. ESKAPE pathogens, which consist of *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species, represent a significant challenge in modern medicine due to their capacity to evade conventional antibiotic treatments, leading to severe healthcare-associated infections (HAIs) with high morbidity and mortality rates (Ramsamy et al., 2018; Dinesh and Karthick, 2018; Li et al., 2022). The term "ESKAPE" was first introduced in 2008 by Rice, highlighting a group of multidrug-resistant (MDR) bacteria that are prevalent in clinical settings (Alsharedeh et al., 2023; Santajit and Indrawattana, 2016). These organisms are identified as critical priority pathogens by the World Health Organization, underscoring the urgent need for effective therapeutic strategies and accounting for approximately two-thirds of all HAIs in the United States (Ramsamy et al., 2018; Dinesh and Karthick, 2018).

The emergence of resistance mechanisms in ESKAPE pathogens is facilitated by biofilm formation, significantly enhancing their resilience against standard antimicrobial therapies (Rizki et al., 2024). Studies have demonstrated that biofilm-producing strains can remain viable on various surfaces for extended periods, contributing to increased transmission rates within healthcare environments (Rizki et al., 2024; Motiwala et al., 2022). Moreover, their virulence factors further complicate treatment, as these pathogens often cause more severe outcomes in infected patients, including prolonged hospital stays and increased healthcare costs (Marturano and Lowery, 2019; Li et al., 2022). The implications of their prevalence necessitate ongoing research and global collaboration to develop targeted interventions that can effectively mitigate their impact on healthcare systems.

In this context, leveraging soil microbes presents a promising strategy for combating these formidable pathogens. Soil microorganisms, particularly those residing in the rhizosphere, have been shown to exert significant antagonistic effects against plant pathogens, potentially providing insights into new biocontrol strategies against human pathogens. For instance, studies reveal that specific groups of soil bacteria such as Oxalobacteraceae and Burkholderiaceae can be particularly effective in suppressing the growth of pathogenic fungi in their environment (Tian and Gao, 2021). These findings suggest that the microbial communities in soil can be harnessed to develop natural products or biological agents capable of disrupting the growth and biofilm formation of ESKAPE pathogens, which are notoriously resilient to conventional antibiotics due to their biofilm-associated resistance mechanisms (Artini et al., 2023).

Recent research has identified antimicrobial compounds produced by environmental bacteria, including those derived from unique habitats, which exhibit effectiveness against ESKAPE pathogens (Artini et al., 2023; Postich and Kiser, 2018). This indicates that not only can soil microbes provide a reservoir for potential new antibiotics, but they can also contribute to biofilm disruption, which is critical for improving treatment outcomes against these pathogens (Artini et al., 2023). Furthermore, the utilization of microbial consortia that include beneficial soil bacteria may enhance their efficacy when applied to clinical settings, promoting a synergistic action against infections involving ESKAPE bacteria (Mulani et al., 2019).

The integration of soil microbes into clinical therapies could also stem from their natural adaptive mechanisms, which facilitate the development of resistance against environmental challenges, making them potentially valuable in overcoming the multidrug resistance exhibited by ESKAPE pathogens (Yi et al., 2021). Moreover, soil microbes could complement existing treatment modalities by offering novel mechanisms of action such as the production of bioactive compounds and metabolites that target the unique molecular pathways of these bacterial pathogens (Pliego et al., 2008). Soil bacteria have emerged as a novel frontier in the battle against ESKAPE pathogens, which are notorious for their multidrug resistance and virulence. This strategy is based on the unique capabilities that certain soil bacteria exhibit, including the production of antimicrobial compounds, competition with pathogenic bacteria, and the potential to disrupt biofilm formation.

Bacteria residing in soil, particularly those with plant growth-promoting properties, can synthesize a variety of antimicrobial agents. For instance, Pseudomonas species, known for their ecological versatility, produce secondary metabolites such as phenazines and antibiotics, which have been shown to exert inhibitory effects on ESKAPE pathogens like *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Artini et al., 2023). These natural products can inhibit bacterial growth and may also interfere with cellular processes critical for pathogenicity, thus mitigating the virulence of these pathogens (Artini et al., 2023). Moreover, soil bacteria actively engage in microbial antagonism, a phenomenon wherein beneficial microbes compete with pathogens for resources and habitats. This competitive dynamic can suppress the growth of ESKAPE pathogens in contaminated environments. For example, the introduction of specific soil microbial communities into clinical settings might enhance biocontrol measures against persistent pathogens through direct competition and the production of inhibitory substances (Mulani et al., 2019).

Mining natural genetic diversity among soil bacteria has unlocked potential new antimicrobial compounds to combat ESKAPE pathogens. Advances in genomics and metagenomics allow for the identification and characterization of unique biosynthetic gene clusters in soil microbes, which may lead to the discovery of new drugs or bioactive molecules that specifically target ESKAPE pathogens (Marie et al., 2024). These bioactive compounds could include novel antibiotics or inhibitors of critical resistance determinants, offering new avenues for therapeutic intervention. To our knowledge, this is the first study conducted on the Fayetteville State University campus aimed at isolating soil-derived bacteria as a potential source of novel antimicrobial compounds. Through this work we hope to identify microorganisms capable of producing antimicrobial agents effective against non-pathogenic safe relatives of ESKAPE pathogens, including *Bacillus subtilis* (safe relative of *E. faecium*), *Staphylococcus epidermidis* (*S. aureus*), *Escherichia coli* (*K. pneumoniae*), *Acinetobacter baylyi* (*A. baumannii*), *Pseudomonas putida* (*P. aeruginosa*), and *Enterobacter aerogenes* (*Enterobacter* spp.). These non-pathogenic surrogates provide a safe and effective initial screening platform, serving as a starting point for identifying antimicrobial-producing isolates prior to testing against clinically relevant ESKAPE pathogens. This study contributes to the growing body of undergraduate-driven research focused on local microbiomes as untapped reservoirs of antibiotic discovery.

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2. Materials and Methods

2.1. Strains and Growth Media

Soil samples were obtained from the campus of Fayetteville State University on June 22, 2023, from topsoil. One gram of soil was resuspended in 9 mL distilled H2O (dH2O), homogenized, and serially diluted in dH2O. 100 µL was spread plated onto Luria Broth (LB) solid media (BD Difco) with 1.5% w/v agar (BD Difco) and 25µg/mL cycloheximide (Fisher Scientific). Single colonies were picked and streaked for isolation. The following safe relatives of the ESKAPE pathogens were used for this study: Bacillus subtilis (ATCC 6051), Staphylococcus epidermidis (14990), Escherichia coli (ATCC 11775), Acinetobacter baylyi (ATCC 33305), Pseudomonas putida (ATCC 23467), and *Enterobacter aerogenes* (ATCC 51697). All strains were cultured at 28°C on LB agar. Incubating at 28°C instead of 37°C reduces the risk of growing potential pathogens and helps promote greater microbial diversity by preventing fast-growing bacteria from outcompeting slower-growing soil species. LB agar is used in experiments to maintain consistency across samples, support the growth of control strains, and provide a standardized baseline for enumerating culturable bacteria.

2.2. Soil Collection

All soil samples were collected from the campus of Fayetteville State University. Sampling sites were selected near vegetated and/or moist areas to maximize microbial diversity, and included coordinates such as 35.0742° N, 78.8925° W; 35°04'33.4" N, 78°53'19.4" W; and 35.069141° N, 78.891485° W. All soil samples had a pH range between 6.0 and 7.0. Soil samples were collected using a sterile metal scoop to dig no more than 6 inches below the surface. This depth corresponds to the topsoil, which is the most fertile layer and contains most of the soil's nutrients. To maximize microbial diversity, dark soil rich in organic material was targeted, particularly near plant roots and areas with moisture. Approximately 5–10 grams of soil were transferred into sterile 50 mL conical tubes. Samples were stored at 4°C to preserve microbial viability. All samples in this study were processed within 24 hours of collection.

2.3. Selection of Soil Isolates

The soil harbors a diverse collection of microbial biodiversity that is rich and dynamic. To best capture a representative sample of this diversity, 63 isolates were selected in this study based on macroscopic characteristics, including size, color, form, margin, elevation, and surface. Table 1 contains characteristic information for the 12 Fayetteville State Isolates (FSI) that were used throughout the study.

2.4. MacConkey Agar Test

MacConkey agar plates were prepared according to the manufacturer's instructions (MP Biomedicals™). Using sterile flat toothpicks, isolates were patched onto the agar surface in a square grid pattern and incubated overnight at 28°C. MacConkey agar is a selective and differential medium that inhibits the growth of Gram-positive bacteria through the presence of crystal violet and bile salts, which disrupt their cell walls. It selectively supports the growth of Gram-negative bacteria and differentiates them based on their ability to ferment lactose. A pH indicator in the medium detects acid production from fermentation, causing lactose-fermenting colonies to appear pink/red. The resulting colony color and growth pattern were recorded the following day to determine Gram identity and fermentation capability.

Table 1. Colony Morphology Characteristics of FSIs. Colony morphology of antimicrobial-producing FSIs was assessed based on visual characteristics observed after incubation on LB agar. Parameters recorded include colony size, color, form, margin, elevation, and surface texture. Observations were made following 24-hour incubation at 28 °C.

Isolate	Size	Color	Form	Margin	Elevation	Surface Texture
FSI 2	Medium	Light yellow	Circular	Entire	Raised	Smooth
FSI 3	Small	Yellow	Circular	Entire	Raised	Smooth
FSI 4	Medium	White	Circular	Entire	Raised	Smooth
FSI 7	Medium	White	Irregular	Entire	Flat	Dry
FSI 8	Small	Dark yellow	Circular	Entire	Convex	Smooth
FSI 9	Large	White	Circular	Entire	Raised	Wrinkled
FSI 10	Large	White	Irregular	Entire	Raised	Smooth
FSI 11	Large	Yellow	Circular	Entire	Raised	Smooth
FSI 12	Small	Yellow	Circular	Entire	Convex	Smooth
FSI 13	Small	Light yellow	Circular	Entire	Raised	Wrinkled
FSI 14	Medium	White	Circular	Entire	Flat	Smooth
FSI 15	Large	White	Circular	Entire	Raised	Dry

2.5. Gram Stain Test

Isolates were grown overnight on LB agar plates at 28°C. A single pure colony was selected and transferred to a clean microscope slide containing a drop of dH_2O. The resulting bacterial smear was air-dried and heat-fixed by passing the slide briefly through the flame of a heat source. The smear was then stained with crystal violet for one minute, followed by rinsing with dH_2O. Gram's iodine was applied for one minute to fix the primary stain and subsequently rinsed off with dH_2O. Decolorization was performed using 95% ethanol for 10 seconds, after which the slide was immediately rinsed with dH_2O. A counterstain of safranin was applied for one minute and rinsed off with dH_2O. The stained slides were blotted with bibulous paper and examined under a light microscope using oil immersion. Cell color and morphology were observed and recorded to determine Gram reaction and cellular characteristics.

2.6. Catalase Test

To assess catalase activity, isolates were grown overnight on LB agar at 28°C. A single pure colony was selected and emulsified in a drop of dH_2O on a clean microscope slide. A drop of 3% hydrogen peroxide (H_2O_2) was then applied directly to the bacterial suspension using a sterile dropper. The presence of catalase was indicated by immediate bubble formation, resulting from the enzymatic breakdown of H_2O_2 into water and oxygen gas. This assay indirectly screens the presence of catalase; a key enzyme associated with the ability of bacteria to detoxify reactive oxygen species.

2.7. Motility Test

Motility agar plates (0.5% agar; Carolina Biological) were prepared according to the manufacturer's instructions and poured into sterile petri dishes, then allowed to cool to a semi-solid consistency. Using a sterile inoculation wire, a single pure bacterial colony from each isolate was aseptically transferred and stab-inoculated into the center of the motility agar. Plates were incubated at 28°C for 24 hours. A qualitative analysis of bacterial motility was documented based on the presence or absence of diffuse growth radiating from the stab site. In addition, a quantitative assessment was performed by measuring the diameter of outward growth in millimeters, conducted in triplicate, and reported as the mean ± standard deviation.

2.8. Antibiotic Resistance Test

Antibiotic susceptibility of bacterial isolates was assessed using the Kirby-Bauer disk diffusion method. Briefly, 1 mL of LB broth was transferred into a sterile 2 mL microcentrifuge tube. A sterile cotton swab was used to collect a single bacterial isolate colony from an overnight culture plate and was then submerged into the LB broth to create a turbid suspension. The same swab was used to uniformly streak the suspension across the entire surface of an LB agar plate to establish a bacterial lawn. Using sterile tweezers, antibiotic-impregnated disks were placed gently onto the surface of the agar and lightly pressed to ensure contact. Antibiotics tested included Ciprofloxacin (5 µg), Neomycin (30 µg), Chloramphenicol (30 µg), Penicillin (10 µg), Streptomycin (10 µg), and Tetracycline (30 µg). A blank disk was included on each plate as a negative control. This procedure was repeated in triplicate for each isolate. Plates were incubated at 28°C for 24 hours. Zones of inhibition were measured in millimeters (mm) and recorded. Interpretations of susceptibility were based on the Clinical and Laboratory Standards Institute (CLSI) guidelines, where isolates were categorized as sensitive (≥ 18 mm), intermediate (14 – 17 mm), or resistant (<13 mm).

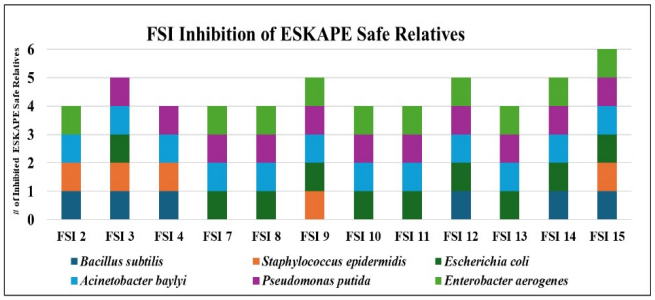
3. Results

3.1. Soil Isolate Activity Against ESKAPE Safe Relative Strains

Soil samples were collected from the campus of Fayetteville State University, a Historically Black College & University (HBCU) located in Fayetteville, North Carolina. Founded in 1867, the university sits on a 156-acre campus that is rich in biodiversity, with an abundance of trees and a small river that runs immediately adjacent to the grounds. These natural features contribute to a variety of microhabitats, increasing diversity of soil microorganisms present. We predict that this environment serves as a reservoir of potentially beneficial soil bacteria that can be utilized to inhibit the safe ESKAPE relatives tested in these experiments. To investigate the potential of antimicrobial molecules producing soil microbiota, soil samples were collected from different locations around Fayetteville State University's campus using 50 mL conical tubes. From each sample, one gram of soil was measured, and serial dilutions from 10-1 to 10-7 were performed to obtain countable and retrievable bacteria colonies. The dilutions are spread onto LB agar plates. To reduce fungal contamination and enrich for bacterial growth, 25 µg/mL of cycloheximide, an antifungal agent, was added to the media. A total of 63 colonies were selected from the serial dilution plates based on morphological characteristics and patched onto fresh plates to allow for individual growth before further testing. After overnight incubation, the 63 Fayetteville State Isolates (FSIs) were screened for antimicrobial activity against the six safe relatives of ESKAPE pathogens to determine their ability to inhibit bacterial growth. This screening was performed in triplicate to ensure reproducibility. For each assay, the safe relative was spread as a lawn across the surface of an agar plate, and FSIs were patched on top in a square grid using a sterile flat toothpick. Plates were incubated overnight at 28°C and examined the following day. Inhibition was indicated by the presence of a clearing zone (zone of inhibition) surrounding the patched colony (Figure 1A). Of the 63 FSIs tested, 12 isolates (19%) exhibited antimicrobial activity against one or more of the six ESKAPE safe relatives, as shown in the stacked bar chart (Figure 1B). Zone of

A.

B.

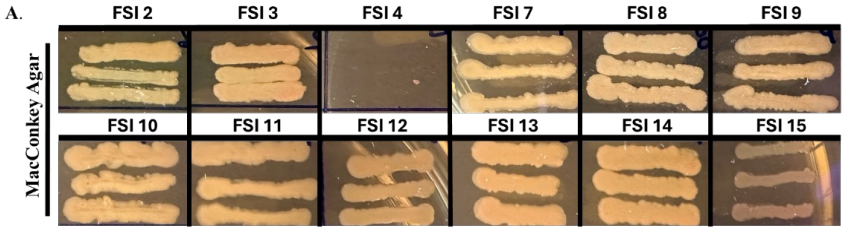


C.

Strains	FSI 2	FSI 3	FSI 4	FSI 7	FSI 8	FSI 9	FSI 10	FSI 11	FSI 12	FSI 13	FSI 14	FSI 15
Bacillus subtilis	17 ± 2	14 ± 1.3	14.3 ± 0.4	NZ	NZ	NZ	NZ	NZ	14.3 ± 0.8	NZ	14 ± 0.6	10.6 ± 0.8
Staphylococcus epidermidis	12.6 ± 1.5	10.3 ± 1.1	4 ± 1.3	NZ	NZ	13.6 ± 0.8	NZ	NZ	NZ	NZ	NZ	14.3 ± 1.1
Escherichia coli	NZ	20.6 ± 0.4	15.6 ± 2.4	10.6 ± 0.4	9.6 ± 0.4	8.6 ± 0.8	8.6 ± 2.2	9.3 ± 0.4	10.3 ± 0.4	5.6 ± 0.8	10.6 ± 0.8	45.6 ± 2.8
Acinetobacter baylyi	12.3 ± 1.1	14 ± 0.6	13 ± 0.6	24.6 ± 0.8	26 ± 0.6	24 ± 0.6	25 ± 2.0	26 ± 0.6	25 ± 0.6	25 ± 0.6	25 ± 0.6	24.3 ± 1.1
Pseudomonas putida	NZ	23.6 ± 0.8	11 ± 0.6	17 ± 0.6	13.3 ± 1.7	16.6 ± 0.4	13 ± 0.6	14.3 ± 1.1	15.3 ± 0.4	14 ± 0.6	12.3 ± 1.1	18 ± 0.6
Enterobacter aerogenes	9.3 ± 1.1	NZ	NZ	20.3 ± 1.1	26.3 ± 1.1	21 ± 0.6	25 ± 0.6	21.6 ± 2.2	25 ± 0.6	20.6 ± 1.5	21.3 ± 0.4	28 ± 8.0

Figure 1. Inhibition of ESKAPE Safe Relatives by FSIs. (A) Representative images of zone of inhibition assays showing antimicrobial activity of FSI strains against safe relatives of ESKAPE pathogens. Clear halos indicate bacterial growth inhibition. (B) Stacked bar chart displaying the number of safe relatives inhibited by each of the 12 active FSIs. (C) Table presenting the mean diameter of inhibition zones (in millimeters ± standard deviation) for each FSI against six ESKAPE safe relatives. All assays were conducted in triplicate and incubated at 28 °C for 24 hours. NZ = No zone of clearance observed.

A.



B.

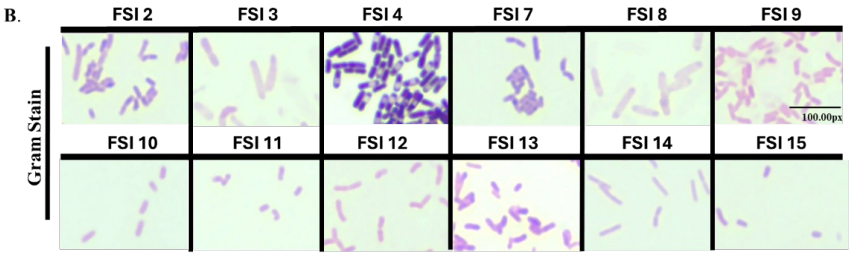


Figure 2. Cell Wall Characterization of FSIs Using MacConkey Agar and Gram Staining. (A) Growth of Fayetteville State Isolates (FSIs) on MacConkey agar. Growth indicates Gram-negative bacteria; lack of growth suggests Gram-positive identity. All isolates that grew were non-lactose fermenters, as evidenced by the yellowish coloration of colonies. (B) Gram staining results for each FSI. Samples were stained using standard procedures. Microscopy was performed at 100× magnification under oil immersion. Pink-stained cells indicate Gram-negative bacteria, while purple-stained cells confirm Gram-positive status (FSI 4). Scale bar in FSI 9 represents 100.00px and applies to all images.

inhibition measurements for each isolate, recorded in triplicate, are reported as mean ± standard deviation in Figure 1C. Notably, Acinetobacter baylyi was inhibited by all 12 active isolates, suggesting it may be particularly susceptible to antimicrobial compounds produced by these soil bacteria. FSI 15 was the only isolate to exhibit broad-spectrum activity, producing zones of inhibition against all six safe relatives. Additionally, FSI 3, FSI 9, FSI 12, and FSI 14 demonstrated inhibitory effects against five out of six safe strains, indicating strong and diverse antimicrobial potential. These results not only highlight the variability in antimicrobial profiles among soil-derived isolates but also emphasize the promising potential of certain isolates, such as FSI 15.

3.2. Phenotypic Characterization of Soil Isolates

After identifying FSI strains that inhibited the ESKAPE pathogen safe relatives, a series of phenotypic characterization assays were performed to further analyze each individual isolate. To determine cell wall differentiation and lactose fermentation capability, MacConkey agar was used as a selective and differential medium for streaking each isolate. Eleven of the twelve FSI isolates were Gram-negative and non-lactose fermenting. FSI 4 did not grow on MacConkey agar, indicating that it is likely Gram-positive (Figure 2A). To confirm the results obtained from MacConkey agar, Gram staining was performed on all isolates. The staining results were consistent with observations from the selective media. Microscopic examination revealed that isolates were either rod- or cocci-shaped and exhibited Gram-negative staining characteristics. Only one isolate, FSI 4, was identified as Gram-positive (Figure 2B). Catalase activity was assessed by applying hydrogen peroxide (H2O2) to bacterial cultures to detect the presence of the catalase enzyme, which breaks down hydrogen peroxide into water and oxygen; the release of oxygen gas results in visible bubble formation, indicating a catalase-positive reaction. Eleven isolates were catalase positive, and FSI 4 was catalase negative (Figure 3A). Motility was assessed using soft agar plates containing 0.5% agar, a lower concentration than standard solid media, which creates a semi-solid consistency that allows motile bacteria to move through the medium. Motile isolates exhibited diffuse, outward growth from the point of inoculation, while non-motile isolates remained confined to the original inoculation site (Figure 3B). Average motility distances, measured in millimeters, indicated that 11 of the 12 isolates exhibited motility, as evidenced by outward diffusion from the inoculation site. Only FSI 4 was non-motile (Figure 3B).

A.

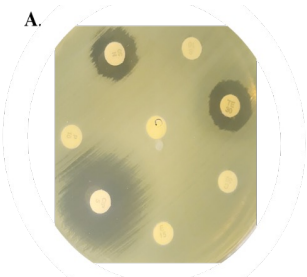
	FSI 2	FSI 3	FSI 4	FSI 7	FSI 8	FSI 9	FSI 10	FSI 11	FSI 12	FSI 13	FSI 14	FSI 15
Catalase (-/+)	+	+	-	+	+	+	+	+	+	+	+	+

B.

	FSI 2	FSI 3	FSI 4	FSI 7	FSI 8	FSI 9	FSI 10	FSI 11	FSI 12	FSI 13	FSI 14	FSI 15
Motility	24.6 ± 2.2	40 ± 2	NM	66.6 ± 4.4	62.6 ± 3.5	66.6 ± 3.7	64.3 ± 3.7	60 ± 2	66.3 ± 2.4	62 ± 2.6	61.6 ± 1.5	62.6 ± 4.2

Figure 3. Catalase Activity and Motility Assays of FSIs. Catalase test results. Representative images show catalase-negative (left; no bubbling) and catalase-positive (right; oxygen bubble formation) reactions upon exposure to hydrogen peroxide. Catalase activity for each FSI is denoted as (+) for positive and (–) for negative. (B) Motility assay results. Representative images depict non-motile (left; confined growth) and motile (right; diffuse outward growth) phenotypes in soft agar (0.5%). The table below shows motility diameters measured in millimeters (mean ± standard deviation) for each FSI. NM = non-motile. All assays were performed in triplicate and incubated at 28 °C for 24 hours.

A.



B.

Antibiotic Name	FSI 2	FSI 3	FSI 4	FSI 7	FSI 8	FSI 9	FSI 10	FSI 11	FSI 12	FSI 13	FSI 14	FSI 15
Ciprofloxacin (5µg)	62.6 ± 1.1	40.3 ± 1.1	55.6 ± 1.1	58.6 ± 1.1	58.6 ± 1.1	47 ± 1.3	46.3 ± 1.1	53.3 ± 1.1	51.6 ± 1.1	57.6 ± 1.5	58.3 ± 0.4	21 ± 0.6
Neomycin (30µg)	32.6 ± 1.1	23.6 ± 1.1	45.3 ± 0.4	31.3 ± 1.1	28.3 ± 1.1	30.6 ± 1.1	26 ± 0.6	28 ± 1.3	28 ± 4.6	27 ± 0.6	29 ± 0	0 ± 0
Penicillin (10µg)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Streptomycin (10µg)	0 ± 0	0 ± 0	43.3 ± 1.5	36 ± 0.6	25.6 ± 1.1	28.3 ± 1.1	28.6 ± 1.1	28 ± 0.6	29.6 ± 1.1	29.6 ± 1.1	33 ± 1.3	0 ± 0
Tetracycline (30µg)	35.3 ± 0.8	24 ± 0.6	42.6 ± 1.7	27 ± 0.6	26.6 ± 1.1	26.6 ± 1.1	24 ± 0.6	24 ± 0.6	25.6 ± 1.1	25 ± 0.6	22 ± 0.6	53.3 ± 1.1
Chloramphenicol (30µg)	0 ± 0	0 ± 0	43.3 ± 1.1	30.6 ± 0.8	24.6 ± 1.7	24 ± 0.6	26.3 ± 1.5	26.6 ± 1.1	24.6 ± 1.1	18.3 ± 0.8	23.3 ± 0.4	33 ± 1.3

Sensitive (S) = zone ≥18 mm; Intermediate (I) = 14–17 mm; Resistant (R) = <13 mm

Figure 4. Antibiotic Susceptibility Profiles of FSIs. Representative image of the Kirby-Bauer disk diffusion assay showing inhibition zones around antibiotic-impregnated disks. (B) Zone of inhibition diameters (mean ± standard deviation, in millimeters) for each FSI tested against six antibiotics. Interpretive breakpoints are based on Clinical and Laboratory Standards Institute (CLSI) guidelines: Sensitive (S) ≥ 18 mm, Intermediate (I) = 14–17 mm, Resistant (R) < 14 mm. All tests were conducted in triplicate and incubated at 28 °C for 24 hours.

3.3. Evaluation of Antibiotic Resistance in Soil Isolates

Testing antibiotic susceptibility is critical in contemporary research due to the increasing prevalence of antibiotic-resistant bacterial infections. Antibiotic susceptibility testing (AST) plays a vital role in determining the effectiveness of specific antibiotics against bacterial pathogens. This is especially important in the context of the global health crisis posed by multi-drug resistant bacteria, which complicate treatment strategies and exacerbate morbidity and mortality rates associated with bacterial infections (Shafrin et al. 2022 and Tuite et al. 2017). To evaluate the antibiotic susceptibility profiles of the FSIs, each isolate was tested against six antibiotics: ciprofloxacin, neomycin, penicillin, streptomycin, tetracycline, and chloramphenicol. For this assay, each FSI was streaked as a lawn on agar plates, followed by placement of antibiotic-impregnated disks. Susceptibility was determined by the presence of a zone of inhibition surrounding the disk, indicating bacterial sensitivity; the absence of a zone indicated resistance (Figure 4A). The average zone of clearance of antibiotic susceptibility for each isolate, in triplicate, is shown in Figure 4B. Several key findings emerged from this analysis. All FSIs were resistant to penicillin, consistent with widespread natural resistance to this antibiotic among environmental bacteria. In contrast, all FSIs were susceptible to tetracycline and ciprofloxacin. Tetracycline targets protein synthesis, while ciprofloxacin inhibits bacterial DNA gyrase; both antibiotics are commonly effective against Gram-positive and Gram-negative bacteria. Notably, only FSI 15 was resistant to neomycin. Streptomycin showed no inhibitory effect on FSI 2, 3, and 15. Additionally, resistance to chloramphenicol was observed in FSI 2 and FSI 3 (Figure 4B).

4. Discussion

In this study, 12 soil isolates from the campus of Fayetteville State University (FSU) were found to inhibit at least one ESKAPE safe relative, out of a total of 63 soil isolates tested, for a rate of 19%. Previous studies have reported rates of about 8% to 12% of soil samples containing antimicrobial-producing organisms (Valderrama et al, 2018). This rate is slightly higher than the average reported in previous studies, suggesting that the FSU landscape, which includes diverse plant life, moist soils, and relatively undisturbed microhabitats, may serve as an ideal environment for the discovery of putative antimicrobial-producing soil microbiota. FSU sits on a 156-acre campus and features a rich diversity of native trees and is bordered by a small river, creating a variety of microhabitats. This diverse and relatively undisturbed landscape likely supports a wide range of microbial communities. As such, educational campuses like FSU may represent underexplored reservoirs of antimicrobial-producing microorganisms, offering unique opportunities for bioprospecting in non-traditional environments.

Several FSIs inhibited more than one safe relative, suggesting that these isolates may produce multiple antimicrobial compounds and/or a broad-spectrum agent. Most of the FSIs were Gram-negative, consistent with reports that soil ecosystems enriched by organic matter, land use, and nutrient availability often support Gram-negative bacterial populations. Literature suggests that Gram-negative bacteria tend to flourish in certain nutrient-rich contexts characterized by their metabolic efficiency in utilizing simpler substrates, often proliferating in nutrient-rich environments due to physiological adaptations, such as the presence of an outer membrane and periplasmic space, which aid in their survival against antimicrobial agents (Shirakawa et al 2019 and Kumar et al 2023).

A correlation was observed among Gram-negative identity, catalase activity, and motility. Specifically, 11 of the 12 isolates demonstrated all three of these phenotypic traits. Catalase activity indicates the presence of the enzyme catalase, which catalyzes the decomposition of hydrogen peroxide (H2O2) into water and oxygen. This may reflect an adaptive advantage conferred by catalase activity, enabling resistance to oxidative stress. This trait is especially beneficial in environments with high levels of reactive oxygen species (ROS), such as those encountered in microbial competition or host immune responses (Masued et al 2024). Motility was observed in the same 11 isolates that were both Gram-negative and catalase positive. Motility contributes to survival in dynamic or unstable soil environments. This trait enables bacteria to migrate away from hostile conditions or toward more favorable microenvironments, further supporting their adaptability and ecological success. Given this pattern, a future hypothesis to test is whether environmental Gram-negative bacteria that are catalase positive and motile are more likely to exhibit antimicrobial activity due to enhanced stress tolerance and competitive fitness. Prior studies have reported that motility and catalase production often co-occur in soil and rhizosphere-associated Gram-negative bacteria, particularly those involved in plant-microbe interactions or secondary metabolite production (Zhang et al., 2018; Lugtenberg and Kamilova, 2009). Testing this hypothesis could help clarify whether this shared phenotype is predictive of antimicrobial potential in soil bacterial communities.

Despite their activity against safe relative of ESKAPE pathogens, majority FSIs remained susceptible to several commercially available antibiotics, with the exception of universal resistance to penicillin which is consistent with natural resistance commonly seen among environmental bacteria. This intrinsic resistance raises important considerations for future therapeutic applications, as there is potential risk for horizontal gene transfer of resistance determinants to pathogenic bacteria, which could complicate their use in drug development. FSI 2 and FSI 3 showed resistance profiles that suggest they may be useful in future studies exploring synergistic interactions with streptomycin and/or chloramphenicol. FSI 15 also exhibited patterns of interest for potential combination with neomycin and/or streptomycin. While these findings are preliminary, they offer a foundation for further investigation into how soil-derived isolates might enhance or complement antibiotic therapies.

This study is not without limitations. Antimicrobial activity was assessed only against non-pathogenic safe relatives of ESKAPE pathogens, which do not fully replicate the resistance mechanisms or virulence of clinical isolates. As such, these results should be interpreted cautiously and not assumed to indicate therapeutic potential against true ESKAPE pathogens. Therefore, it is premature to conclude that these isolates produce novel antimicrobial agents, as the bioactive compounds responsible for the observed inhibitor activity have not yet been isolated, identified, or chemically characterized. Additionally, molecular identification of the isolates using 16S rRNA gene sequencing has not yet been conducted, which limits taxonomic classification at this stage.

Future work will involve testing the most promising FSIs against clinically relevant, multidrug-resistant ESKAPE pathogens to assess whether the observed inhibitory activity extends to pathogenic strains. Additionally, studies will focus on the extraction, purification, and chemical characterization of the active compounds, followed by mechanism of action analysis. 16S rRNA gene sequencing will also be conducted to identify the antimicrobial-producing isolates at the genus level. Lastly, evaluating potential additive or synergistic interactions between bacterial isolates and commercially available antibiotics may provide insight into how such combinations could be leveraged in the fight against antibiotic resistant infections.

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References

Alsharedeh RH, A Yehya, OB Yonis, O Alameri, and N Alshraiedeh (2023). Characterization of ESKAPE pathogens in urinary tract infections among Jordanian patients. The Journal of Infection in Developing Countries 17(03): 374-380.

Artini M, R Papa, G Vrenna, M Trecca, I Paris, C D'Angelo, et al. (2023). Antarctic marine bacteria as a source of anti-biofilm molecules to combat ESKAPE pathogens. Antibiotics 12(10): 1556.

Baltz RH (2006). Marcel Faber Roundtable: is our antibiotic pipeline unproductive because of starvation, constipation or lack of inspiration? Journal of Industrial Microbiology and Biotechnology 33(7): 507-513.

Centers for Disease Control and Prevention (CDC). Antibiotic Resistance Threats in the United States, 2019. Atlanta, GA: U.S. Department of Health and Human Services, CDC; 2019

Dinesh K and M Karthick (2018). A study on ESKAPE pathogens the bad bug with no drug. Tropical Journal of Pathology & Microbiology 4(2): 134-138.

Hessle C, B Andersson, and AE Wold (2000). Gram-positive bacteria are potent inducers of monocytic interleukin-12 (IL-12) while gram-negative bacteria preferentially stimulate IL-10 production. Infection and immunity 68(6): 3581-3586.

Hughes D and A Karlén (2014). Discovery and preclinical development of new antibiotics. Upsala journal of medical sciences 119(2): 162-169.

Kumar KA, RD Singh, and SK Sahu (2023). Influence of land use on soil microbial communities of sub-Himalayas of India: insights from phospholipid fatty acid profiles, ribosomal intergenic spacer profiles, soil enzymes, and carbon pools. Agroforestry Systems 97(8): 1571-1585.

Lewis K (2013). Platforms for antibiotic discovery. Nature Reviews Drug Discovery 12(5): 371-387.

Li J, X Su, J Li, W Wu, C Wu, O Guo, et al. (2022). The association of organ preservation fluid pathogens with early infection-related events after kidney transplantation. Diagnostics 12(9): 2248.

Lugtenberg B and F Kamilova (2009). Plant-Growth-Promoting Rhizobacteria. Annual Review of Microbiology 63: 541.

Marie JS, C Mays, B Guo, TS Radniecki, J Waite-Cusic, and T Navab-Daneshmand (2024). Impact of biosolids amendment on the soil resistome and microbiome–a greenhouse study. bioRxiv, 2024-09.

Marturano JE and TJ Lowery (2019, December). ESKAPE pathogens in bloodstream infections are associated with higher cost and mortality but can be predicted using diagnoses upon admission. In Open Forum Infectious Diseases (Vol. 6, No. 12, p. ofz503). US: Oxford University Press.

Masued MS, SU Rehman, MM Shah, L Zafar, R Ahmed, MT Khan, et al. (2024). Exploring Fresh Lettuce (Lactuca sativa) as a Dairy-Free Probiotic Source. Journal of Microbiological Sciences 3(01): 1-11.

Motiwala T, Q Mthethwa, I Achilonu, and T Khoza (2022). ESKAPE pathogens: looking at Clp ATPases as potential drug targets. Antibiotics 11(9): 1218.

Mulani MS, EE Kamble, SN Kumkar, MS Tawre, and KR Pardesi (2019). Emerging strategies to combat ESKAPE pathogens in the era of antimicrobial resistance: a review. Frontiers in Microbiology, 10, 539.

Opimakh S (2023). Selman Abraham Waksman, Born in Ukraine – An outstanding scientist, a nobel laureate, the discoverer of streptomycin and the modern era of antituberculosis chemotherapy. Ukraine Pulmonology Journal 31: 65-73. https://doi.org/10.31215/2306-4927-2023-31-4-65-73.

Pliego C, S De Weert, G Lamers, A De Vicente, G Bloemberg, FM Cazorla, and C Ramos (2008). Two similar enhanced root-colonizing Pseudomonas strains differ largely in their colonization strategies of avocado roots and Rosellinia necatrix hyphae. Environmental Microbiology 10(12): 3295-3304.

Postich CA and KB Kiser (2018). Characterization of Antibiotic producing Bacterium Isolated From Anthill Sediment With Activity Against ESKAPE Pathogens. Fine Focus 4(2): 189-201.

Ramsamy Y, SY Essack, B Sartorius, M Patel, and KP Mlisana (2018). Antibiotic resistance trends of ESKAPE pathogens in Kwazulu-Natal, South Africa: a five-year retrospective analysis. African Journal of Laboratory Medicine, 7(2): 1-8.

Ribeiro da Cunha B, LP Fonseca, and CR Calado (2019). Antibiotic discovery: where have we come from, where do we go? Antibiotics 8(2): 45.

Rizki LP, IK Murni, AT Aman, and T Nuryastuti (2024). Environmental Metagenomic Analysis of" ESKAPE" Pathogens in the Pediatric Intensive Care Unit of General Hospital Yogyakarta Indonesia. Progress In Microbes & Molecular Biology, 7(1). https://doi.org/10.36877/pmmb.a0000398.

Santajit S and N Indrawattana (2016). Mechanisms of antimicrobial resistance in ESKAPE pathogens. BioMed research international 2016(1): 2475067.

Shafrin J, A Marijam, AV Joshi, FS Mitrani-Gold, K Everson, R Tuly, et al. (2022). Economic burden of antibiotic-not-susceptible isolates in uncomplicated urinary tract infection: Analysis of a US integrated delivery network database. Antimicrobial Resistance & Infection Control, 11(1).

Shirakawa M, I Uehara, and M Tanaka (2019). Mycorrhizosphere bacterial communities and their sensitivity to antibacterial activity of ectomycorrhizal fungi. Microbes and Environments 34(2): 191-198.

Tian Y and K Gao (2021). Responses of Soil Microbial Communities to the Invasion of Fusarium Oxysporum and Screening for Fusarium Antagonistic Microorganisms From Pathogenic Soil. Research Square. https://doi.org/10.21203/rs.3.rs-227497/v1.

Tsuchido Y, R Horiuchi, T Hashimoto, K Ishihara, N Kanzawa, and T Hayashita (2019). Rapid and selective discrimination of Gram-positive and Gram-negative bacteria by boronic acid-modified poly (amidoamine) dendrimer. Analytical Chemistry 91(6): 3929-3935.

Tuite AR, TL Gift, HW Chesson, K Hsu, JA Salomon, and YH Grad (2017). Impact of rapid susceptibility testing and antibiotic selection strategy on the emergence and spread of antibiotic resistance in gonorrhea. The Journal of Infectious Diseases 216(9): 1141-1149.

Valderrama M, B González-Zorn, P Pablo, R Diez-Orejas, T Fernández-Acero, J Gil-Serna, L Juan, H Martín, M Molina, BF Navarro-García, Patiño, J Pla, D Prieto, C Rodríguez, E Román, A Sanz-Santamaría, M Silóniz, M Suárez, C Vázquez, and V Cid (2018). Educating in antimicrobial resistance awareness: adaptation of the small world initiative program to service-learning. Fems Microbiology Letters 365(17).

Wang W, Y Weng, T Luo, Q Wang, G Yang, and Y Jin (2023). Antimicrobial and the resistances in the environment: Ecological and health risks, influencing factors, and mitigation strategies. Toxics 11(2): 185.

Yi G, SN Riduan, A Armugam, JT Ong, PY Hon, MY Abdad, et al. (2021). Nanostructured copper surface kills ESKAPE pathogens and viruses in minutes. ChemMedChem 16(23): 3553-3558.

Zhang Q, X Gao, Y Ren, X Ding, J Qiu, N Li, F Zeng, and Z Chu (2018). Improvement of Verticillium wilt resistance by applying arbuscular mycorrhizal fungi to a cotton variety with high symbiotic efficiency under field conditions. Int. J. Mol. Sci. 2018, 19, 241.