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Dear reader,

We are delighted to present the fifth annual volume of the *Anne Arundel Community College Journal of Emerging Scholarship* — a milestone that reflects five years of growing student curiosity, rigorous inquiry, and scholarly achievement at AACC.

In 2026, as technological advances, environmental challenges, and societal shifts continue to reshape our world at an unprecedented pace, the value of undergraduate research has never been clearer. Our student authors have tackled timely and timeless questions — exploring innovations in science and technology, examining pressing social and environmental issues, and contributing fresh perspectives across the humanities and beyond. Their work demonstrates that meaningful scholarship begins not in distant laboratories or elite institutions, but right here in our classrooms, libraries, and community.

What makes this journal possible is the remarkable collaboration between talented students and dedicated faculty mentors. Every manuscript has undergone a thoughtful review process, helping students develop essential skills in critical thinking, persistence, and professional communication that will serve them well whether they transfer to four-year institutions or enter the workforce.

We hope that readers — fellow students, faculty, staff, community members, and external scholars — will find inspiration in these pages. May the research presented here spark new questions, encourage future submissions, and affirm that every voice has the potential to contribute to the ever-growing body of knowledge.

Sincerely,
The 2025–2026 Editorial Board

Saachi Chopra, Bethany Bayer, and Dr. Sandy Fox-Moon

Nature's Secret Weapon: Identification and Classification of an Unknown Organism with Natural Antimicrobial Activity

Abstract

Antimicrobial resistance (AMR) is a global healthcare issue that threatens lives by enabling pathogens to evade currently available treatment options, making the discovery or development of new therapies critical. An alternative to traditional antimicrobial drug development is surveying environmental microorganisms that produce novel antimicrobial agents. In this study, an unknown organism isolated from a shoe exhibited antimicrobial activity on Sabouraud Dextrose Agar (SDA). The classification of the organism was based on cultural characteristics, staining (simple, Gram, and endospore stains), biochemical testing, and genetic analysis. The unknown organism was identified as a Gram-positive endospore-forming bacillus based on staining. Polymerase chain reaction (PCR) using universal 16S rRNA primers amplified a 1500-base-pair DNA fragment, and Nucleotide BLAST analysis of the PCR DNA fragment identified the unknown organism as a member of the *Bacillus* genus. Biochemical tests produced a metabolic profile that most closely matched the unknown organism to *Bacillus velezensis*. Cross-streaking with Gram-negative and Gram-positive bacteria displayed potential inhibition for *Enterobacter cloacae* ATCC 23355, *Bacillus megaterium* ATCC 14581,

Key words

antimicrobial resistance (AMR)

antimicrobial agent

Bacillus

unknown organism

Faculty Mentor

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Staphylococcus aureus ATCC 25923, and *Staphylococcus saprophyticus* ATCC 15305, suggesting antimicrobial activity. Future research includes verifying the identity of the unknown organism and the isolation and characterization of the antimicrobial agent.

INTRODUCTION

Global antimicrobial resistance (AMR) is an escalating threat responsible for an estimated 39 million deaths as of 2024 (Naghavi et al., 2024) and is a stress on an overburdened healthcare system, costing approximately $55 billion annually in the United States (Aslam et al., 2024). Limited regulation and fewer treatment options in developing and low-income countries (Obaigbe & Elikwu, 2023), and the misuse of antibiotics, such as overprescription (Llor & Bjerrum, 2014), failure to complete drug regimens (Oliveira et al., 2024) and feeding sub-therapeutic doses to livestock for growth promotion and disease prevention (Pandey et al., 2024), are major contributors to the rise of antimicrobial-resistant pathogens. Other factors contributing to AMR include poor hospital infection control and greater reliance on antibiotics (CDC, 2024). Patients face heightened risk from recurrent, prolonged infections caused by drug-resistant pathogens, resulting in extended hospital stays that strain healthcare staff and increase overall hospital costs (Shafrin et al., 2022). The environment can play an instrumental role in AMR through public contamination of medication (used, expired, or unused) and transnational travel (groundwater, migratory animals, climate change, or human movement) (Bokhary et al., 2021), which spreads drug-resistant pathogens (Collignon & Beggs, 2025).

Drug resistance in microorganisms occurs extrinsically and intrinsically. Drug resistance arises extrinsically when bacteria acquire random genetic mutations or obtain resistance genes from other organisms through horizontal gene transfer, including transformation, conjugation, and transduction (Belay et al., 2024).

These mechanisms enable bacteria to remove antimicrobial agents via efflux pumps, inactivate or modify drug targets, often assisted by mobile genetic elements such as plasmids and transposons (Belay, et al., 2024). Lastly, the intrinsic or inherent resistance exhibited by drug-resistant organisms is attributable to multiple protective determinants, including the absence of cell walls, presence of double-membrane cell envelopes, mechanisms that confer protection against their own antimicrobial compounds, and the ability to form biofilms (Usui et al., 2023).

Biofilms are microbial communities that embed themselves in a self-created extracellular matrix. These structures protect microorganisms from immune responses and antimicrobial drugs. Drug resistance within biofilms occurs through several mechanisms. First, antibiotics slowly or incompletely penetrate biofilms, providing natural protection (Sharma et al., 2019). Slow-growing cells in biofilms are naturally resistant because many drugs target metabolism, protein synthesis, and cell wall synthesis. Lastly, organisms in biofilms are in close proximity allowing for enhanced horizontal transfer of drug resistance genes (Uruén et al., 2020). Biofilms adhere to surfaces and play a crucial role in dental plaque formation (Marsh, 2006), foggy contact lenses (Voinescu et al., 2024), hospital-acquired infections (Assefa & Amare, 2022), livestock infections (Abdullahi et al., 2016), and foodborne illnesses (Liu et al., 2023). With the emergence and persistence of more resistant strains and as commonly used antiseptics and antibiotics lose efficacy against biofilms (Falconer et al., 2025), one approach to developing antimicrobial agents is to design drugs, including novel drugs that target new cellular structures and processes, such as biofilms. However, because the development of novel antimicrobial agents is an inherently labor-intensive endeavor, it is imperative that alternative strategies be pursued to advance antibiotic innovation.

Efforts have been focused on discovering novel antimicrobial

compounds to respond to the global healthcare crisis of AMR (Miethke et al., 2021). One strategy to discover novel antibiotics is to screen the environment (soil, water) for natural sources of antimicrobial agents. Soil is a complex living mixture consisting of minerals, air, water, organic matter, and organisms (Pecsi et al., 2024), including a variety of bacteria and fungi. Bacteria, the most prevalent soil microorganisms, play a role in breaking down organic material, recycling nutrients, and supporting soil fertility (Wang et al., 2024). In addition, some soil bacteria (Actinomycetes family, *Streptomyces*, *Bacillus*, *Amycolatopsis*) and fungi (*Penicillium*, *Acremonium*) produce antimicrobial agents to protect themselves and to compete against other soil organisms for scarce nutrients and space (Clardy et al., 2009). The Actinomycetes family of soil bacteria produces most of the naturally occurring antibiotics (Clardy et al., 2009). Some soil bacteria produce bacteriocins, small bioactive peptides that assist in microbial competition, and, indirectly, influence the spread of antibiotic resistance through resistance gene co-selection (Pino-Hurtado, 2023). Soil bacteria, such as *Bacillus* and *Brevibacillus* species, produce biocontrol compounds with antimicrobial properties that protect plants from disease (Pino-Hurtado, 2023). An example of a recently discovered bacteriocidal compound produced by soil bacteria is teixobactin. Teixobactin, produced by the soil bacterium *Eleftheria terrae*, is a novel antibiotic that targets some Gram-positive bacteria by disorganizing the cell envelope by binding to precursors of peptidoglycan and teichoic acid (Piddock, 2015, Hussein et al., 2020). Teixobactin affects *Staphylococcus aureus* strains, including highly resistant vancomycin-resistant *S. aureus* (VRSA) (Hussein et al., 2020). Currently, teixobactin is in late-stage preclinical development (NovoBiotic Pharmaceuticals, 2025). Although antimicrobial compounds like teixobactin have demonstrated efficacy and show promise as a potential therapy, antibiotic development has declined over the past few decades due to low profitability, high research costs, and

stringent regulatory hurdles (World Health Organization, 2022). In addition, conventional drug discovery approaches have proven inadequate for developing new antibiotics, as they cannot keep pace with the rapid evolution of AMR (Farha et al., 2025).

The discovery of new antibiotics produced by soil bacteria has other applications including the classroom setting. Educational exploration programs like MicroMundo and Tiny Earth utilize citizen science projects to allow students to explore soil microbiota for antimicrobial activity. This raises awareness of AMR and improves student understanding of global issues (Pino-Hurtado, 2023).

Due to frequent exposure to diverse environments, such as soil, shoes can harbor a variety of organisms, including bacteria, viruses, and fungi (Sangwan, 2025). This study focused on the identification and classification of an unknown microorganism with potential antimicrobial activity isolated from a swab of a shoe using staining, biochemical, and genetic techniques.

MATERIALS AND METHODS

Sample Collection and Isolation

As part of a classroom lab exploring ubiquity, a sample was collected from the shoe outsole of a General Microbiology student using a sterile cotton swab and was inoculated on a Sabouraud Dextrose Agar (SDA) plate. A variety of organisms grew on the agar plate, with one colony that had a clearing around it, indicating possible microbial inhibition (Figure 1A). This unknown organism was selected for this study because of its potential antimicrobial activity. The colony containing the unknown organism (Figure 1B) was streaked for isolation on SDA and Trypticase Soy Agar (TSA) plates to obtain pure cultures. A 24-hour culture of the unknown organism grown statically in Nutrient Trypticase Soy (NTS) Broth at 37°C was used to inoculate various morphological and biochemical tests.

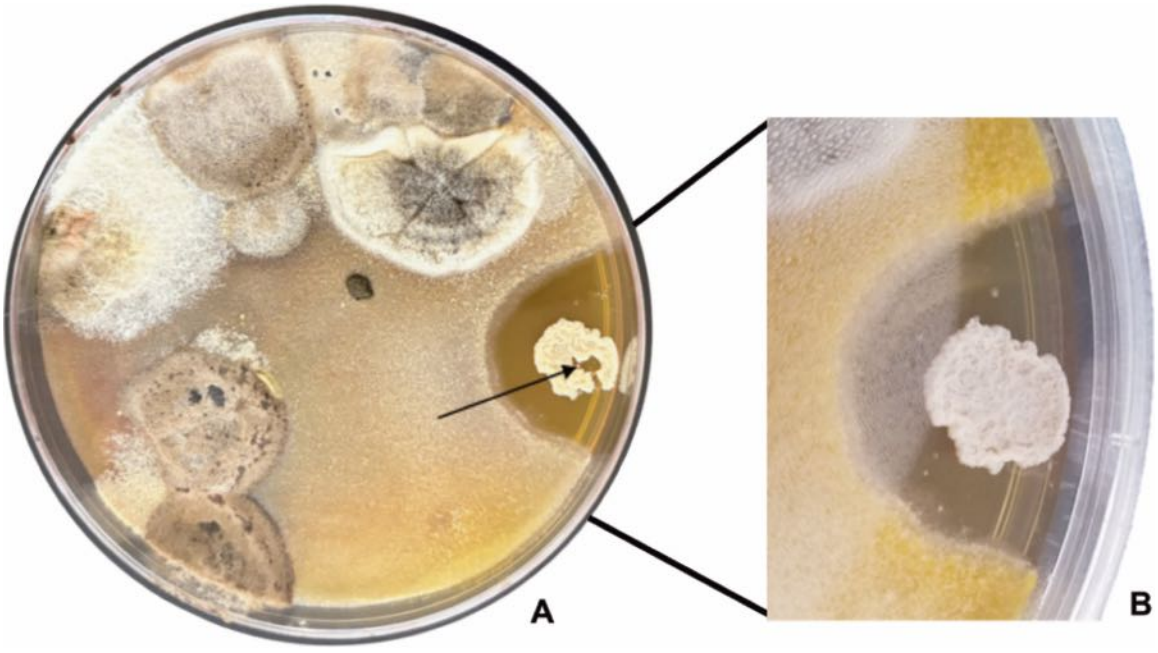


FIGURE 1

Original Agar Plate of the Unknown Organism. A Sabouraud Dextrose Agar (SDA) plate was inoculated with a swab from the bottom of a shoe. The black arrow indicates the unknown organism (Figure 1A). The clearing within the colony is where the sample was taken (Figure 1A). The clearing around the colony indicated potential antimicrobial activity (Figure 1B).

Unknown Identification

To identify the unknown microorganism, the results were compared using the second edition of Bergey's Manual (De Vos et al., 2009). Colony morphology, Gram reaction, and biochemical test results were used to narrow down the possible genus. Features were cross-referenced with charts and descriptions provided in Bergey's Manual, which aligned with the most probable species.

Cultural Characteristics

To investigate the unknown organism's growth characteristics and for further testing, samples of the overnight culture in NTS Broth were inoculated on both SDA and TSA plates. The plates were incubated at 37°C for 24 hours to promote microbial growth, and then the colonies were analyzed.

Morphology and Staining

Several staining procedures were performed on colonies grown on SDA and TSA. Simple staining with methylene blue determined basic cell type (bacterial or fungal), cell morphology, size,

and arrangement. Gram staining was performed to determine the unknown organism's cell wall type. Lastly, endospore staining was conducted to identify spore formation. Motility was analyzed by inoculating the unknown organism into Motility Agar containing 2,3,5-Triphenyltetrazolium Chloride (TTC) using a sterile inoculating needle, incubating at 37°C for 24 hours, and observing the growth pattern. The result was confirmed using Sulfur Indole Motility (SIM) Agar.

Oxygen Requirements

The oxygen requirement of the unknown organism was determined by inoculating Fluid Thioglycolate Media (FTM) with a sterile inoculating loop, incubating at 37°C for 24 hours, and observing the growth at the inoculation site.

Biochemical Testing

Colonies of the unknown organism were inoculated into multiple culture media for biochemical testing including nitrate reduction (nitrate broth with reagent A [sulfanilic acid] and B [α-naphthylamine]), citrate utilization (Simmons Citrate Agar), sugar fermentation (Phenol Red Glucose, and Sucrose Broths), along with mixed-acid and 2,3-butanediol production through glucose fermentation using Methyl Red Voges-Proskauer (MRVP) Broth (methyl red reagent and Barritt's A [α-naphthol in ethanol] and B [40% potassium hydroxide]). Production of enzymes, including urease (urea broth), cysteine desulfurase (SIM Agar), DNase (DNase Agar), amylase (Starch Agar), and casease (Skim Milk Agar), were examined. All tests were incubated at 37°C for 24 hours, except Simmons Citrate Agar, MRVP, and urea broth, which were incubated for 48 hours at 37°C. The presence of catalase was determined by adding a sample of bacteria directly onto slides with diluted hydrogen peroxide. Biochemical test results were evaluated based on color changes, gas production, enzymatic

activity, or growth inhibition as noted elsewhere (Leboffe & Pierce, 2011).

PCR Amplification

The classification of the unknown organism was determined by amplifying and sequencing the 16S rRNA gene via colony polymerase chain reaction (PCR). A single colony of the unknown organism was used as the template DNA. The primers used are as follows (Ag et al., 2014): universal 16S rRNA 8F forward primer (5'-AGTTGATCCTGGCTCAG-3') and universal reverse primer 1492R (5'-ACCTTGTTACGACTT-3'). The PCR reaction mixture contained 25 µL of 2.0X Taq RED Master Mix Kit (Apex), 0.5 µL of forward and reverse primers (100 µM), a single colony and 24 µL of deionized water, for a final volume of 50 µL. The following protocol was used for amplification: Denature at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 2 minutes, then a final extension at 72°C for 5 minutes.

Gel Electrophoresis

Gel electrophoresis was used to verify the presence and size of DNA samples after each PCR procedure and for gel extraction of the PCR fragment. Using a standard protocol, 5 µL of the unknown DNA samples were loaded on a 1% agarose gel (0.2 g agarose [Fisherbrand] in 20 mL 1X TAE buffer [Promega]) at 150 volts for 1 hour (Apogee Electrophoresis, n.d.). The DNA ladders (1 kb, 100 bp, [New England Biolabs NEB]) were prepared by adding 7 µL of the DNA ladder to 3 µL 6X Gel Loading Dye, Purple (NEB). To visualize the DNA fragments, the agarose gels containing the samples and DNA ladders were stained with Ethidium Bromide solution for 10 minutes on a shaker at 65 rpm. The predicted size of the 16S rRNA gene PCR fragment was 1500 bp (Sacchi et al., 2002).

Gel Extraction and Sequencing of the PCR Fragments

The Qiagen Gel Extraction Kit was used according to a standard protocol, with the addition of isopropanol (QIAGEN, 2020). The PCR fragments were eluted with sterile deionized water to a final volume of 50 µL. The gel-extracted PCR fragments from the unknown organism were sequenced (GENEWIZ, Azenta Life Sciences), using the universal 16S rRNA gene forward primers. The partial DNA nucleotide sequences were compared to known DNA sequences using Nucleotide BLAST (National Center for Biotechnology Information [NCBI]).

Cross-streaking

A cross-streak method from Dev et al. (2013) was used for the preliminary screening of antimicrobial activity. A single line of test bacteria was streaked on SDA and TSA plates. The plates were then incubated at 37°C for 24 hours, followed by perpendicular streaking with different Gram-negative (*Escherichia coli*, *Klebsiella oxytoca* ATCC 8724, *Enterobacter cloacae* ATCC 23355, *Proteus mirabilis* ATCC 7002, *Citrobacter freundii* ATCC 8090, and *Morganella morganii* ATCC 25830) and Gram-positive bacteria (*Bacillus megaterium* ATCC 14581, *Bacillus subtilis* ATCC 6051, *Staphylococcus aureus* ATCC 25923, *Staphylococcus saprophyticus* ATCC 15305, *Mycobacterium smegmatis* ATCC 14468, and *Enterococcus faecalis*). The cross-streaked agar plates were then incubated at 37°C for an additional 24 hours. The antimicrobial cross-streak tests were evaluated based on the presence or absence of inhibition zones. Replicates were completed on both plates to confirm results.

RESULTS

Cultural Characteristics

The colony morphology of the unknown organism was observed on SDA and TSA plates. Opaque, off-white-colored colonies were observed on both plates. After 24 hours, the unknown organism

grown on SDA plates exhibited more robust growth and larger colonies (~ 4.5 mm) (Figures 2A, 2B) as compared to their growth on TSA plates (~ 2.3 mm) (Figures 2C, 2D). The configuration and elevation were irregular and umbonate, while the margin was either undulated (SDA) or filamentous (TSA) (Figures 2B, Figure 2D). After 24 hours, when the unknown organism was grown statically in NTS Broth, a distinctive pellicle formed at the top (Figure 2E).

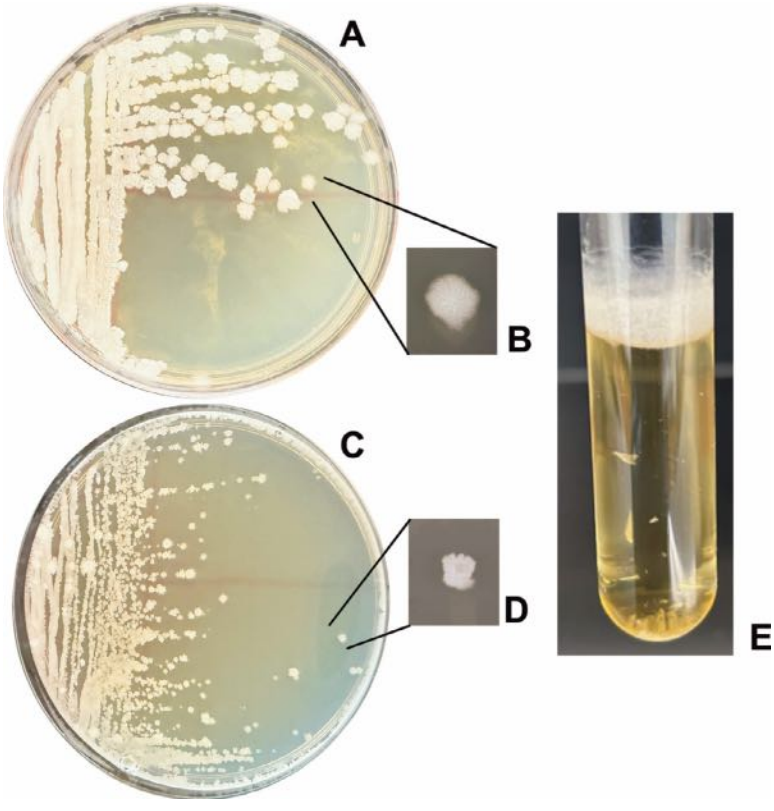


FIGURE 2

Cultural Characteristics of the Unknown Organism. Streak plates were inoculated with the unknown organism on Sabouraud Dextrose Agar (SDA, Figures 2A and 2B) and Trypticase Soy Agar (TSA, Figures 2C and 2D) and incubated at 37°C for 24 hours. Pellicle formation was noted when the unknown organism was grown statically in Nutrient Trypticase Soy (NTS) Broth after 24 hours (Figure 2E).

Cell Morphology and Staining Properties

The simple stain with methylene blue revealed that the unknown organism was a bacterium rather than a fungus (larger, filamentous structures) (Figures 3A and 3B). The arrangement and morphology of the unknown organism at 100X were single bacilli or rod-shaped cells, with an average size of 1µm width x 5 µm length from TSA (Figure 3A) or 1µm width x 3 µm length if derived

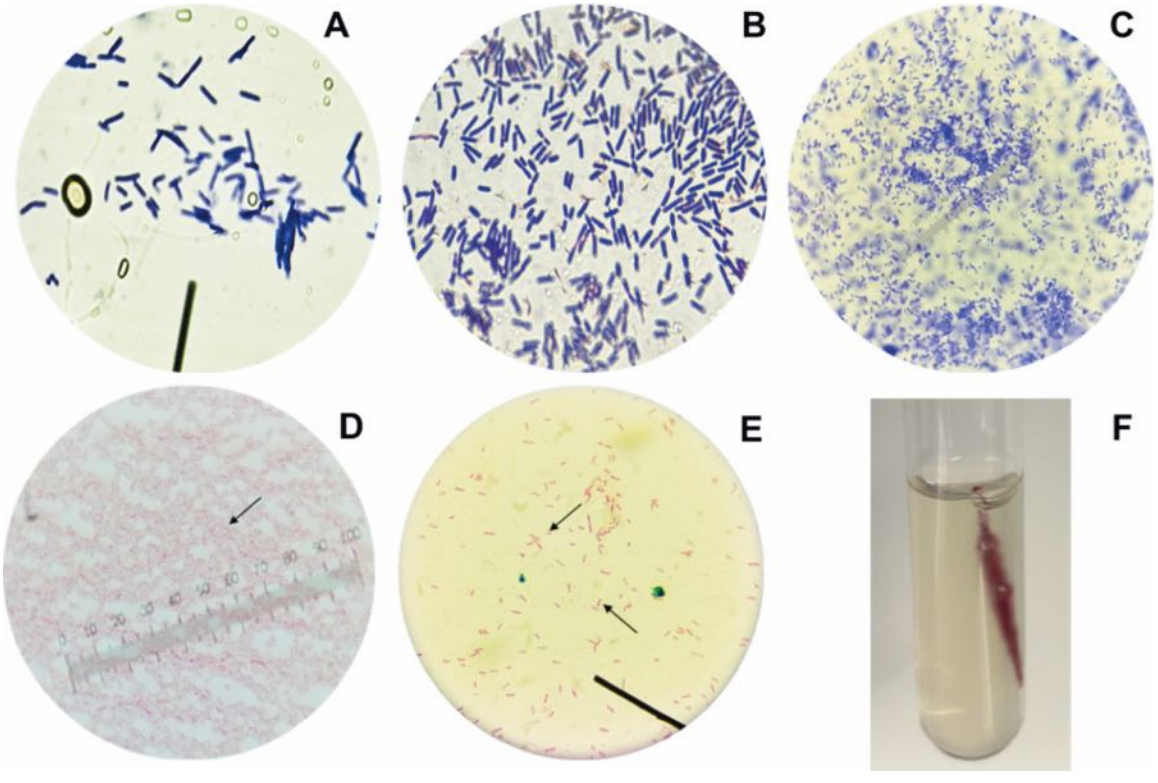
from SDA (Figure 3B). Gram-staining revealed Gram-positive bacilli (Figure 3C) with clear regions indicating possible endospore presence (Not pictured). Endospore staining determined that the unknown organism produced centrally positioned (Figure 3E, black arrows), elliptical (oval) endospores (Figures 3D and 3E, black arrows). The unknown organism grown on TSA plates produced large clusters of endospores with minimal vegetative cells (Figure 3D), while the unknown organism grown on SDA plates exhibited minimal sporulation (Figure 3E). Based on the TTC Motility Agar (88% of samples tested) and SIM Agar, the unknown organism is predicted to be nonmotile (Figure 3F, Table 1).

Oxygen Requirements and Biochemical Testing

Oxygen requirements for the unknown organism was determined to be a strict aerobe based on their growth at the top of the FTM tube (Table 1). Biochemical tests determined that the unknown

FIGURE 3

Microscopic and Motility Morphologies of the Unknown Antimicrobial Agent. Methylene blue simple stain (Figures 3A [Trypticase Soy Agar TSA] and 3B [Sabouraud Dextrose Agar SDA]), Gram stain (Figure 3C), and endospore stains (Figures 3D [TSA] and 3E [SDA]) of the unknown organism viewed at 100X. Elliptical (oval) clearings representing endospores are indicated by the black arrow (Figure 3D). Minimal endospore formation was observed with centrally positioned endospores noted by the arrows (Figure 3E). Growth of the unknown organism in TTC Motility Agar was observed as a distinct red line (Figure 3F).



organism was positive for nitrate reduction (88%, Figure 4A), casein hydrolysis (Skim Milk, 100%, Figure 4B), glucose fermentation (88%) including 2,3-butanediol fermentation (VP, 88%), citrate utilization (75%, Figure 4C), and catalase (80%, Figure 4D), (Table 1). The unknown organism was negative for mixed acid fermentation (MP, 100%), urea hydrolysis (100%), sucrose fermentation (75%), DNase production (100%), and starch hydrolysis (80%) (Table 1).

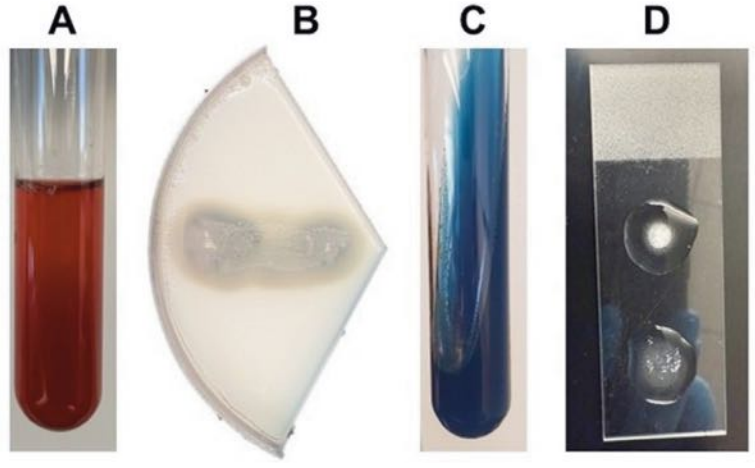
TABLE 1 *Biochemical Results of the Unknown Organism**

**Numbers represent the percentage are based on 8 rounds (light gray) or 5 rounds (dark gray) of biochemical testing*

Motility	Citrate	Nitrate	MR	VP	Urea	FTM	Glucose	Sucrose	Catalase	DNAse	Starch	Skim Milk
- (88)	+ (75)	+ (88)	- (100)	+ (88)	- (100)	Aerobe (100)	+ (88)	- (75)	**+ (80)**	**- (100)**	**- (80)**	**+ (100)**

FIGURE 4

Positive Test Results of the Unknown Organism. Examples of positive biochemical tests include tests for nitrate reduction (Figure 4A), casein hydrolysis (Figure 4B), citrate utilization (Figure 4C), and catalase production (Figure 4D).



Unknown Identification

Based on microscopic observations (Gram-positive endospore-former) and biochemical testing, the organism was most likely a bacterium from the genus *Bacillus*. The second edition of Bergey's Manual (De Vos et al., 2009) was consulted for comparison between 95 known *Bacillus* species. Results indicate a strong similarity

between the unknown bacterium and the species *Bacillus anthracis*, *Bacillus gibsonii*, and *Bacillus niacini* (reclassified as *Neobacillus niacini*) (Table 2).

TABLE 2 *Comparison of the Unknown Organism to Other Bacillus Species Based on Bergey's Manual*

	Unknown	*B. amyloliquefaciens*	*B. anthracis*	*B. gibsonii*	*B. niacini*	*B. subtilis*	*B. velezensis*
Motility	-	+	-		d	+	+
> 1.0 µm	+	-	+	-	v	-	+
Ellipsoidal	+	+	+	+	+	+	+
Catalase	+		+		+	+	+
Aerobic growth	+	+	+	+	+	+	+
Voges-Proskauer	+	+	+		d	+	+
Casein	+	+	+	+	-	+	+
Starch	-	+	+	-	d	+	+
Citrate	+	+	d^i		d^i	+	d^i
Nitrate	+	+	+	d	+	+	+
Glucose	+			+			+
DNAse	-	d					v
Urea	-	-	-		d	-	-

Legend	
Red	doesn't align
Green	aligns
Yellow	possibility
	no data available
+	>85% positive
-	negative, 0-15% positive
d	strains give different reactions
v	variations w/in strains
d^i	citrate tests create various results

Genetic Identification

To verify the identity of the unknown organism, PCR was conducted using universal primers targeting the 16S rRNA gene. A 1500 bp PCR fragment was observed for the unknown bacterium and the control, *Bacillus subtilis* (Figure 5). Fainter 3000 bp bands

were noted in the unknown bacterium samples (Figure 5). The gel-extracted 1500 bp fragments were subsequently sequenced. Of the four PCR samples sent, only one sample from the unknown organism yielded a result. The partial DNA nucleotide sequence (Figure 6A) and the E-values and Percentage Identical Nucleotide BLAST results (Figure 6B) of the sequenced PCR sample revealed that the PCR DNA sequence of the unknown organism was similar to the 16S rRNA gene sequence from *Bacillus subtilis*, *Bacillus amyloliquefaciens*, and *Bacillus velezensis*.

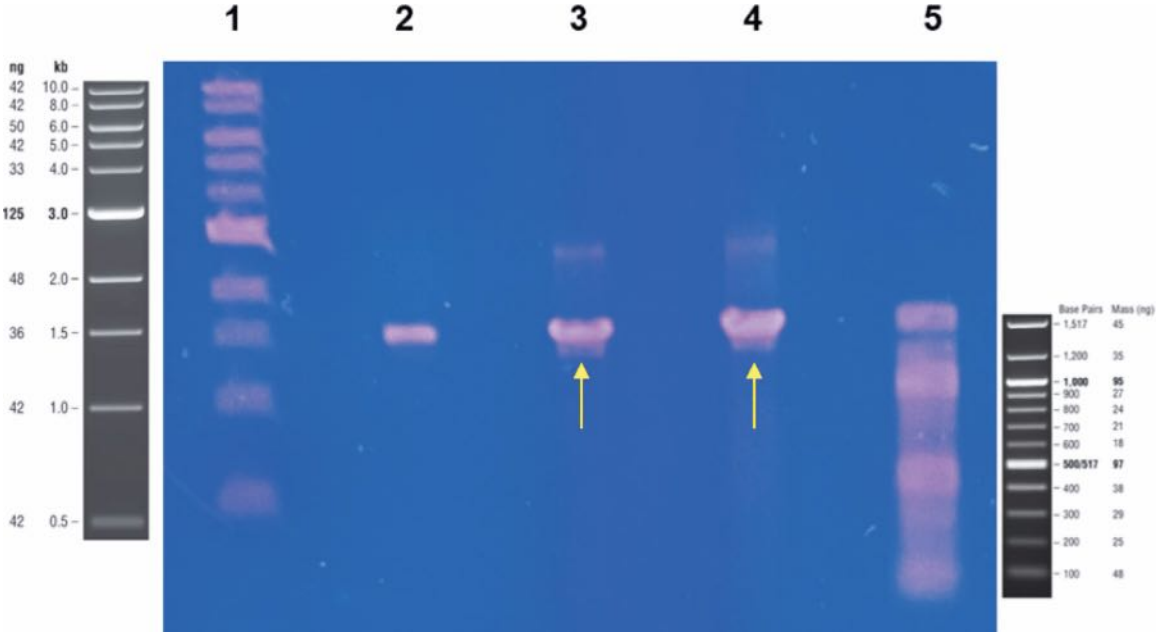


FIGURE 5 *Gel Electrophoresis Results of the Unknown Organism PCR Samples Before Gel Extraction. The unknown organism PCR DNA fragments were run on a 1% agarose gel at 150 volts for 1 hour and visualized using ethidium bromide (Lanes 3 and 4). Fragments of approximately 1500 bp (yellow arrows) and 3000 bp were present in the unknown organism PCR samples. A control PCR sample using the same universal 16S rRNA gene primers with Bacillus subtilis DNA (1500 bp, Lane 2), a 1 kb ladder (Lane 1), and a 100 bp ladder (Lane 5) were included. The 1 kB (Left) and 100 bp (Right) DNA Ladder images are from the NEB Website.*

A. Unknown Organism PCR DNA Sequence | 386 bp

NNNNNNNNNNNNNNNNNNGNNNGTCNAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAG
CGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAA
ACCGGGGCTAATACCGGATGGTTGTCTGAACCGCATGGTTCAGACATAAAAGGTGGCTTC
NGCTACCACTTACANATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACC
ATTGCGACNATGCGTAGCCGACCTNTTTTTTTGATCGGCCACACTGGGACTGAGACACGG
CCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGANGG
ACCAANAATAAANTGANTGANGAANG

B. Nucleotide BLAST results (E-values and Percentage Identical) for the 386 bp fragment

Bacterial Strain Name	E-value	Percentage Identical
***B. subtilis* strain E78**	1e-157	96.20%
***B. amyloliquefaciens* strain Ak59**	4e-157	95.92%
***B. subtilis* strain GB 6 SAUDI**	2e-155	95.63%
***B. velezensis* strain B2**	2e-155	95.63%
***B. amyloliquefaciens* strain HBAU S69758**	2e-155	95.63%
***B. velezensis* strain TPB20**	2e-155	95.63%
***B. velezensis* strain Bac214**	2e-155	95.63%
***B. velezensis* strain PHP1601**	2e-155	95.63%

FIGURE 6 *Partial DNA Nucleotide Sequence of Unknown Organism PCR Fragments and Nucleotide BLAST Results. One partial Unknown Organism PCR DNA sequence was compared to known DNA sequences using Nucleotide BLAST (NCBI) for identification. The partial DNA nucleotide sequence (Figure 6A) and Nucleotide BLAST results (E-values and Percentage Identical) for the 386 bp fragment (Figure 6B) are shown.*

Cross-streaking

One Gram-negative (*E. cloacae* ATCC 23355) and three Gram-positive (*B. megaterium* ATCC 14581, *S. aureus* ATCC 25923, *S. saprophyticus* ATCC 15305) bacteria consistently exhibited decreased growth when cross streaked with the unknown organism on SDA (Figures 7A and 8A) and TSA (Figures 7B and 8B) plates. Occasional inhibition was noted for some of the test strains (*E. coli*, *M. smegmatis* ATCC 14468, *E. faecalis*) on SDA plates (Figures 7A and 8A).

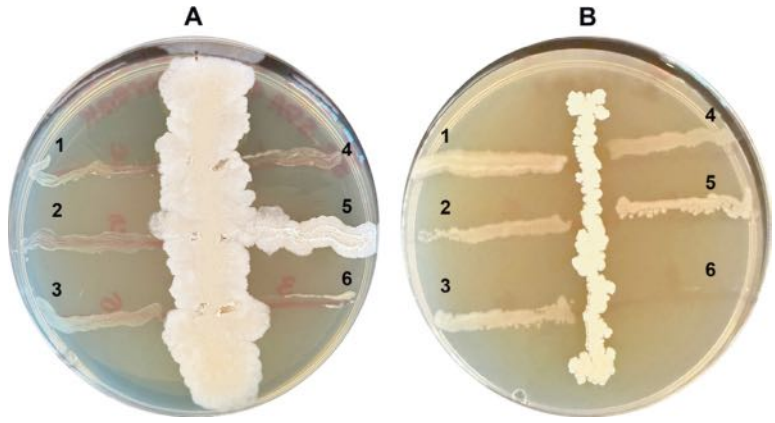


FIGURE 7 *Cross-streaking with Gram-negative Bacteria to Test Antimicrobial Activity of the Unknown Organism. The unknown organism was streaked vertically and Gram-negative test bacteria (Proteus mirabilis ATCC 7002 [1], Citrobacter freundii ATCC 8090 [2], Morganella morganii ATCC 25830 [3], Escherichia coli [4], Klebsiella oxytoca ATCC 8724 [5], Enterobacter cloacae ATCC 23355 [6]) were streaked perpendicular to the unknown organism streak. The decreased growth of E. cloacae ATCC 23355 is noted on both SDA (Figure 7A) and TSA (Figure 7B) plates, with varied growth of E. coli (Not shown).*

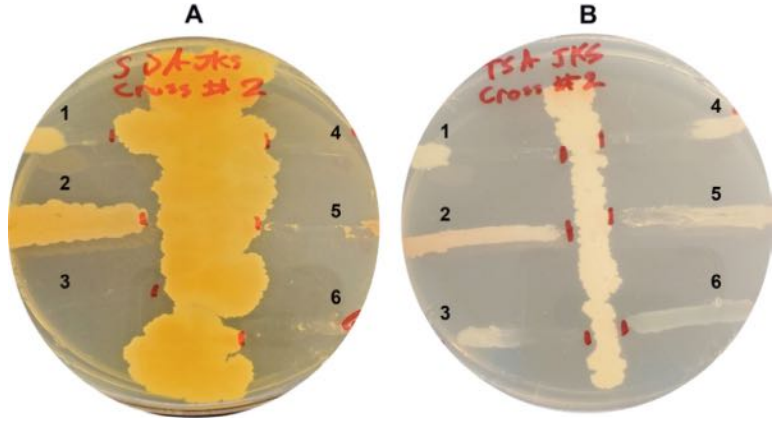


FIGURE 8 *Cross-streaking with Gram-positive Bacteria to Test Antimicrobial Activity of the Unknown Organism. The unknown organism was streaked vertically and Gram-positive test bacteria (Bacillus megaterium ATCC 14581 [1], Bacillus subtilis ATCC 6051 [2], Staphylococcus aureus ATCC 25923 [3], Staphylococcus saprophyticus ATCC 15305 [4], Mycobacterium smegmatis ATCC 14468 [5], Enterococcus faecalis [6]) were streaked perpendicular to the unknown organism streak. The decreased growth of B. megaterium ATCC 14581, S. aureus ATCC 25923, and S. saprophyticus ATCC 15305 was noted on both SDA (Figure 8A) and TSA (Figure 8B) plates, with varied growth of M. smegmatis ATCC 14468 and E. faecalis on SDA (Figure 8A).*

DISCUSSION

The focus of this study was to identify and classify an unknown organism with potential antimicrobial activity from a swab of a shoe outsole using staining, biochemical, and genetic techniques.

After obtaining a pure culture, it was observed that the unknown organism had increased growth on SDA (Figure 2A) as compared to TSA (Figure 2C), suggesting it was a more favorable environment (higher glucose levels and lower pH) for the unknown organism, as compared to the neutral pH, higher protein conditions of TSA. The acidic environment of SDA inhibits most bacterial growth while promoting the growth of fungi and yeasts (Aryal, 2015), suggesting the unknown organism could be a fungus. However, the colony morphology of the unknown organism was more representative of potential bacterial colonies (smaller, flat) than fungal colonies (large, hairy) (Madiga et al., 2012). A notable observation was that the unknown organism's growth preference led to overgrowth on some SDA plates, complicating the interpretation of results during cross-streaking which could have been caused by variability in SDA culture preparation or uneven inoculation. After reviewing the current literature, *B. velezensis* is commonly grown on Luria-Bertani (LB) Agar or nutrient agar, not SDA or TSA, so a direct comparison of colony morphology cannot be made due to differences in nutrient composition of the various culture media. However, colony morphology of the unknown organism is similar to colonies of *B. velezensis* grown on nutrient agar (Fu et al., 2025). *B. velezensis* is known to produce pellicles when grown in culture media which was observed for the unknown organism when grown statically in TSB after 24 hours (Shao et al., 2024). A study by Sun et al. (2022) showed that *B. velezensis* formed robust biofilms when co-cultured with *Pseudomonas stutzeri* in TSB, indicating that these organisms can form pellicles when grown in TSB.

Simple staining with methylene blue revealed that the

unknown organism was bacterial due to the presence of small single bacilli (1 µm width x 3-5 µm length) (Figure 3) as compared to fungal spores (3-40 µm) and filamentous molds (microns to meters long) (Jeong et al., 2022). Fungi, especially yeasts, can appear as slightly larger (3-10 µm) Gram-positive or Gram-negative round organisms (Barenfanger & Drake, 2001). Gram-positive rod-shaped organisms were observed after Gram-staining, indicating that the unknown organism was likely a single bacillus, based on microscopic morphology and arrangement. The unknown organism's cell size varied under the microscope, depending on the medium from which the sample came (SDA or TSA). Since the unknown organism forms endospores when grown on TSA (Figure 3D), as determined by the endospore stain, perhaps the larger organisms are preparing to form endospores, which increases their size. *B. velezensis* is known to be a Gram-positive endospore-forming single bacillus, forming elliptical endospores, with a cell size between 1.8 to 2.7 µm in length, 0.5 to 0.64 µm in width (Podstawka, n.d., Torres et al, 2026, Chen et al., 2021). The cell sizes we observed for *B. velezensis* varied from the known literature which could be due to the fact we used media that is not commonly used to culture this organism. Nutrient composition of culture media influences organism cell size (Yao et al., 2012).

To narrow down possible candidates, the initial cultural and microscopic findings were used. Based on these initial findings (Gram-positive endospore-forming bacilli with the ability to form pellicles in TSB Broth), *Bacillus* species appeared as the best option, as these bacteria are Gram-positive, endospore-forming aerobic or facultatively anaerobic bacilli (Turnbull, 1996), with the ability to form pellicles on liquid media (Figure 2E) (Kobayashi, 2007).

Inconsistent results were found for various morphological and biochemical tests, including oxygen requirements and motility. The unknown organism grew at the top of the FTM tube, suggesting that the unknown organism is an obligate or strict

aerobe. However, when evaluating the Motility Agar, growth was noted from the stab line to the bottom of the tube, indicating that the unknown organism could be a facultative anaerobe. The aerobic nature of the unknown organism was confirmed with positive result of the catalase test (Table 1). These differences could be due to human errors in inoculation or interpretation. As the unknown organism forms pellicles, pellicle-forming bacteria can release surfactants that prevent floating cells from mixing in liquid culture media, a phenomenon which is observed for aerobes and facultative anaerobes (Rune et al., 2024). This could explain why the unknown organism was not found throughout the FTM tube. *B. velezensis* has been shown to have aerobic and facultative anaerobic metabolism (Cheng et al., 2024), so further studies can be completed to verify the unknown organism oxygen requirements such as growth in an anaerobic jar.

For motility, the unknown organism was nonmotile in TTC Motility Agar and SIM Agar (Figure 3F, Table 1), while *B. velezensis* is known to be motile (Torres et al, 2026). Pellicle formation is typically observed in actively swimming bacteria (Kunoh et al., 2022). The lack of motility could be explained by the physical properties of the media, such as oxygen levels and nutritional composition, which can influence the flagellar expression in bacterial motility (Chu & Zhuang, 2022).

Based on the initial microscopic, morphological and biochemical results and using the second edition of Bergey's Manual as a reference (De Vos et al., 2009), three *Bacillus* species were considered the best candidates: *B. anthracis*, *B. gibsonii*, and *B. niacini*, with the latter most closely aligned with the unknown organism (Table 2) (De Vos et al., 2009). No candidate was a 100% match as variance in results occurs due to typical differences within a strain, differences within a test, or variance possible within a species (De Vos et al., 2009).

To identify the *Bacillus* species of the unknown organism,

genetic analysis was performed. The successful amplification of the 16S rRNA gene (~1500 bp) using universal 16S rRNA-specific primers supports the genetic identification of the unknown organism as a *Bacillus* species. Faint 3000 bp bands were visible, in addition to the 1500 bp fragments, in the unknown organism PCR samples. This could be due to nonspecific binding of the primers to the genomic DNA, resulting in unintended amplification at a random genomic location (Lorenz, 2012). The Expect Values or E-values from the Nucleotide BLAST analysis were low (< 10e−100), indicating a highly significant match between the unknown organism PCR DNA sequence and *Bacillus* strains listed (*B. subtilis*, *B. amyloliquefaciens*, and *B. velezensis*), suggesting homology (NCBI, 2024, QIAGEN, 2021). Re-evaluation of the phylogenetic results after the genetic analysis did not match the initial predictions (Figure 6), which were based on the second edition of *Bergey's Manual of Systematics of Archaea and Bacteria* (Logan & De Vos, 2015). While having the most comprehensive data on *Bacillus* species, the second edition of Bergey's Manual does not contain newly discovered species such as *B. velezensis*, which was discovered in 2005 (Logan & De Vos, 2015). After re-evaluation, the unknown organism was most likely *B. velezensis*, based on the morphological, biochemical and genetic analyses. *B. velezensis* is known not to be biochemically uniform and produce heterogeneous results, especially for enzymatic activity (Brutscher et al., 2024). This could explain some of the inconsistencies found in the study.

A decrease in growth was noted for *E. cloacae* ATCC 23355, *S. aureus* ATCC 25923, *S. saprophyticus* ATCC 15305, *E. coli*, *B. megaterium* ATCC 14581, and *E. faecalis* in the cross-streaking assay (Figures 7 and 8). *B. velezensis* could be a potential candidate as this organism is commonly used as a biocontrol agent in agriculture to promote growth and to protect against plant pathogens (Ning et al., 2025). This soil-dwelling organism is known to produce several antimicrobial compounds, bacillomycin-D, fengycins,

bacillibactin, bacilysin, macrolactin, bacillaene, difficidin, and iturin (Zhou et al., 2022, Keshmirshekan et al., 2024). *B. velezensis* has been shown to have activity towards a variety of Gram-positive (*S. aureus* [Afroj et al., 2021], *B. megaterium* [Zhong, 2024], *E. faecalis* [Byun et al., 2023]) and Gram-negative bacteria (*E. cloacae* [Liang et al., 2021]) (Baharudin et al., 2021, Byun et al., 2023). It is plausible since *B. velezensis* is a soil-dwelling bacterium it has evolved resistance mechanisms to maintain dominance in microbial communities by competing with common soil bacteria for space and nutrients (Mullis et al., 2019, Rabbee et al., 2019).

Most of the susceptible bacteria tested in this study have pathogenic strains. Thus, the potential antimicrobial agent or agents produced by the unknown organism may have broad-spectrum activity, making it a potential therapy for a range of infections. However, it must be taken into consideration that the growth decreases observed may be due to the growth conditions in SDA (acidic, high-sugar). Performing an agar diffusion assay with the antimicrobial agent could determine if this is the case.

Some of the test strains were resistant to the antimicrobial activity of the unknown bacterium. The resistance observed for *B. subtilis* ATCC 6051 may reflect microbial competitive tolerance through lipopeptide production or coexistence mechanisms such as spatial segregation, allowing this bacterium to persist in the presence of other organisms (Kobayashi, 2021). It is also possible that the unknown bacterium is *B. subtilis*, in which case it would have natural resistance to the antimicrobial agent.

Future Research

Future research should focus on additional tests, such as growth curves to determine when the antimicrobial activity is produced, growth assessments at various pH levels, enzymatic activity, and hydrolysis substrate testing. Efforts should be directed toward characterizing the antimicrobial compound, investigating its mode of

action, and evaluating its effectiveness against a broader spectrum of pathogens, particularly those resistant to conventional antibiotics. Microbial interaction studies through expanded cross-streak analysis, agar plug diffusion, and co-culture media growth can help identify suppression, inhibition, or coexistence with other microbial strains. Additional studies could determine the genetic element (plasmid, chromosome, transposon) that harbors the gene(s) responsible for the unknown agent and proteins needed to produce it.

While *B. velezensis* is known as a biocontrol agent and its ability to produce a variety of antimicrobial compounds, there is limited research on this bacterium in other areas. Studies have shown that *B. velezensis* can be found in the human gut and has antimicrobial activity, making it a candidate for probiotics (Byun et al., 2023). Since biofilms are commonly associated with gut flora (de Vos, 2015) and *B. velezensis* is known to produce pellicles (Shao et al., 2024), future studies could include biofilm studies of this organism to see if culture media affects biofilm formation, what genes are important for biofilm formation, and the association of biofilm formation and antimicrobial production. Future studies can include looking for other soil-dwelling organisms with antimicrobial activity in the local area to isolate potential novel antimicrobial agents.

CONCLUSION

As AMR remains a critical global health threat, continued exploration of environmental microbiomes will be essential in identifying new antimicrobial agents. Macroscopic and microscopic analyses were performed on an unknown organism with antimicrobial activity that originated from a shoe swab. Staining (simple, Gram, and endospore), biochemical testing, and genetic analysis gave a preliminary identification of the unknown organism as the bacterium *Bacillus velezensis*. Certain Gram-positive and Gram-negative

bacteria were found to be susceptible to the antimicrobial activity of the unknown organism as determined by the cross-streaking assay. Understanding the metabolic functions and investigating underexplored enzymatic and biochemical properties may provide insight for pharmaceutical and clinical applications. Future research efforts are required to verify the identity of the unknown organism and define its potential applications, while discovering new antimicrobial agents from natural sources.

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