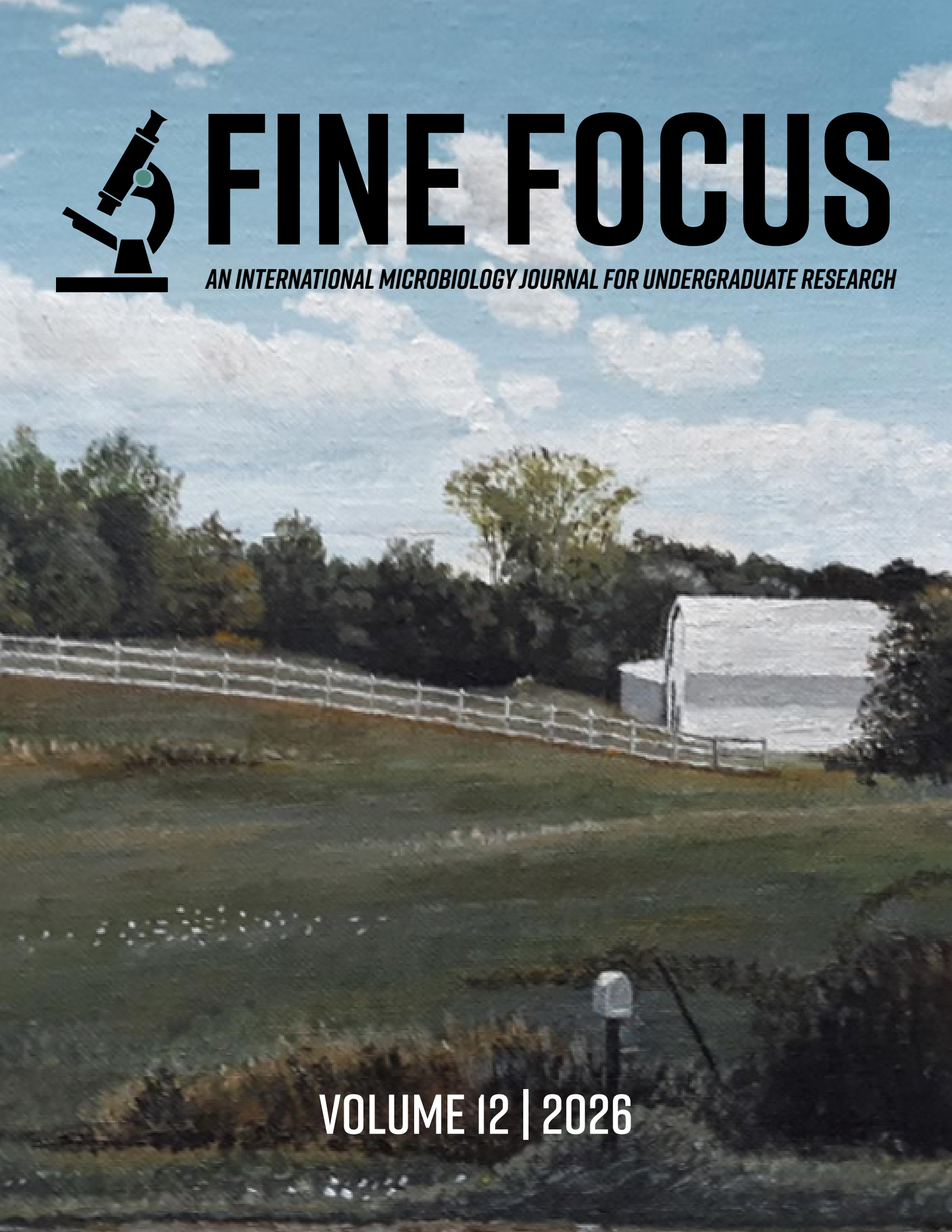

# FINE FOCUS

AN INTERNATIONAL MICROBIOLOGY JOURNAL FOR UNDERGRADUATE RESEARCH

VOLUME 12 | 2026

# FINE FOCUS

## AN INTERNATIONAL MICROBIOLOGY JOURNAL FOR UNDERGRADUATE RESEARCH

We publish original research by undergraduate students in microbiology. This includes works in all microbiological specialties and microbiology education.

We are a web and print journal dedicated to showcasing the research of undergraduate students, internationally, in all fields of microbiology. *Fine Focus* is managed entirely by undergraduate students from production to print.

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**COVER ART**

Front and Inside Covers, article headers: *View from the Front Door*, original acrylic piece painted by John Pichtel.

Back Cover: American Goldfinch exploring the Lake of the Woods in Geneva, Indiana USA, near the Limberlost wetland area and the home of author and naturalist Gene Stratton-Porter. Photo credit: Bill Hubbard.

VOLUME 12 | 2026

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# Objective Lens:
# *In Memoriam:* John Pichtel (1957-2026)

The community of environmental scientists lost a dear colleague recently: Professor John Pichtel passed away on January 9, 2026. John retired from Ball State University in 2024 from what is now the School of Earth, Atmosphere, and Sustainability. During his 37-year career at BSU, John's research focused on developing improved methods for handling hazardous materials, from explosive residues in contaminated brownfield sites to the phytoremediation of heavy-metal contaminants in wetlands and other watershed environments.

Over his long and prolific career, John served as a visiting scholar in Italy, Scotland, Ireland, and Finland. John led several extensive field studies in environmental science and served as the director of the BSU Australia Center multiple times. His list of awards and honors is extensive, including two Fulbright Scholar awards, and his publications are staggering in both volume and quality. During his time at BSU, he published more than 80 papers and four books on topics ranging from environmental remediation to microplastics to weapons of mass destruction. In 2004, he helped create the Emergency Management and Homeland Security (EMHS) program at Ball State University, serving as its director for twenty years. His legacy is felt by many well beyond the campus community.

John and I worked together on many occasions, including my serving on the dissertation committee of his last doctoral student a few years ago. In earlier years, we collaborated on a project involving a metagenomics analysis of petroleum-contaminated soils, publishing together across disciplines. Over time, John became a trusted and respected colleague and friend. His approach to mentoring graduate students was something I always tried to emulate, knowing that no one was as gifted at

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inspiring students as he was.

John was an inherently curious scholar and lifelong learner with diverse interests. Since his retirement, he and I had discussed his honing Italian and French language skills, mastering chess, traveling, planting wildflowers, and his progress on the novel he was writing. John was also an avid, quite talented painter, with a wide variety of themes in his works on canvas. During our last lunch together, a few weeks before his passing, he shared some of his paintings with me and, with the blessing of his wife, Theresa, I selected one to use as the cover on this issue of *Fine Focus*.

Featuring this acrylic pastoral scene, entitled *View from the Front Door*, on our cover seems an appropriate way for me to say, "Thank you, John," for inspiring me as a scientist and mentor, and for so many great memories of working together all these years. I am reminded of a quote by Ernest Hemingway in his 1940 novel, *For Whom the Bell Tolls*: "Today is only one day in all the days that will ever be. But what will happen in all the other days that will ever come can depend on what you do today." To me, this captures John's legacy, which will live on in the countless students he mentored and the dozens of scientists with whom he collaborated.

John, this issue of *Fine Focus* is dedicated to you. It is fitting that multiple articles in this issue are applied and/or environmental microbiology studies, and I would like to think that you would be pleased to see this. Thank you for your friendship. You will be sorely missed.

-John McKillip, Managing Editor

# Unveiling the Microbes of the World's Largest Flowers

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## ABSTRACT

The plant family Rafflesiaceae is known for producing the largest flowers in the world. They are solely parasitic to the plant genus *Tetrastigma* of the grape family. Interestingly, members of Rafflesiaceae are the only plants we know so far that have lost their chloroplast genome. Rafflesiaceae is comprised of three genera: *Rafflesia, Sapria,* and *Rhizanthes.* Members of Rafflesiaceae are restricted to the fast-disappearing tropical forests of Southeast Asia and are endangered. It is virtually unknown how Rafflesiaceae infects its hosts. This makes Rafflesiaceae very difficult to conserve and propagate. However, bacterial endophytes have shown to play a major role in promoting the growth of plants. In this study, we characterized and analyzed the associated microbiome of Rafflesiaceae, particularly *Rafflesia speciosa* and *Sapria himalayana* to find "microbial markers" of Rafflesiaceae infection. It was hypothesized that *Rafflesia* and its *Tetrastigma* host will have similar microbiomes given their intimate connection, however, results across different taxonomic levels demonstrate that *Rafflesia* buds have a distinct microbial endophyte composition and possessed some unique genera compared to its infected hosts. Its microbiome was also diverse compared to *Sapria*'s. Non-host *Tetrastigma* spp. possessed unique bacteria that may be antagonistic to *Rafflesia* infection. This study unveiled the symbiotic microbes of *Rafflesia* and *Sapria,* which may have potential horticultural application in propagating the world's largest flowers.

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## LITERATURE REVIEW

### BACKGROUND ON THE FAMILY RAFFLESIACEAE

The plant family Rafflesiaceae is known for producing the largest flowers in the world, up to a meter in diameter, but unlike most plants, members of this family do not have stems, roots nor leaves and lack the ability to photosynthesize. They are solely parasitic to the plant genus *Tetrastigma* of the grape family (Nikolov & Davis, 2017). However, not all *Tetrastigma* species can be parasitized. Interestingly, members of Rafflesiaceae are the only plants we know so far that have lost their chloroplast genome, the set of genes that purportedly defines plants (Molina et al., 2014; Cai et al., 2021). Rafflesiaceae belongs to the order Malpighiales, and it is comprised of three genera: *Rafflesia* R.Br. ex Thomson, *Sapria* Griff, and *Rhizanthes* Dumort. *Rafflesia* contains 41 species, *Rhizanthes* contains 4 species, and *Sapria* contains 4 species as well (Plants of the World Online). The first *Rafflesia* species (*R. arnoldii*) was discovered in 1818 by Sir Stamford Raffles and Dr. Joseph Arnold when they embarked on a journey to explore the Sumatran island in Bengkulu (Brown, 1821). Two of the four *Rhizanthes* (*Rh. lowii* and *Rh. zippelii*) species were found in the early nineteenth century in Indonesia by German botanist Alexander Zippelius and were then described by Karl Ludwig von Blume (Wicaksono et al., 2022). The first *Sapria* species (*S. himalayana* Griff) was described in 1836 by the British botanist William Griffith afrom his north-east Indian collection (Dorji et al., 2022). Unfortunately, all members of Rafflesiaceae are rare, restricted to the fast-disappearing tropical forests of Southeast Asia, and are thus endangered. They are a charismatic icon of conservation, hailed as the "panda of the plant world".

### LIFE CYCLE OF RAFFLESIACEAE

As a holoparasite, Rafflesiaceae siphons organic and inorganic nutrients from their hosts and reside inside its host as strands of cells also known as endophytes. It has been observed that a *Rafflesia* endophyte can grow inside its host plant for approximately 2-3 years (Hidayati et al., 2000; Wicaksono et al., 2021). As it transitions to its flowering stage, the strands of cells become a mass of cells called a protocorm that eventually emerges as a flower bud from the inside of the woody host (Nikolov & Davis, 2017). As the flower blooms, it emits a rotting meat type of smell that attracts pollinators such as carrion flies. The pollination process consists of a fly entering a male flower, exiting with pollen grains attached to its body, and transferring it to a female flower (Beaman et al., 1988). After successful pollination, it takes several months for the fruits to form. The fruit then dehisces revealing millions of seeds. The fruit produces a rancid coconut meat odor that attracts multiple insect visitors. Although little is known about the seed dispersal of Rafflesiaceae, it is thought that the seeds can be dispersed by different kinds of wild animals like shrews, squirrels, elephants, and ants (Wicaksono et al., 2021).

### PROPAGATION AND CONSERVATION EFFORTS

It is virtually unknown how Rafflesiaceae infects its hosts (Wicaksono et al., 2021). This makes Rafflesiaceae very difficult to conserve and propagate. It has never been cultivated outside of its native range. Since 2015, Molina et al (2017) have been trying to propagate *Rafflesia* species from Southeast Asia at the United States Botanic Garden (USBG) by importing *Rafflesia*-infected host cuttings and *Rafflesia* seeds. Molina et al (2017) have tried to induce germination of *Rafflesia speciosa* seeds with the use of different plant growth regulators (PGRs) including extracts from the host plant *Tetrastigma*. The results showed that there was no germination in the *Rafflesia speciosa* seeds. However, there were four PGRs (GA4+7, KAR1, kinetin, & BRA) that induced germination in wildflower seeds that were used as controls, and these PGRs were combined and applied on the *Rafflesia speciosa* seeds but this treatment was also unsuccessful. Researchers have also tried to stimulate germination on other species such as *Rafflesia patma* Blume, but the *in vitro* germination assays on *Rafflesia* species remains a continuing challenge (Wicaksono et al., 2016). Molina et al (2017) explained that the unsuccessful attempt in germination is a result of not knowing the simulating host chemicals and/or that the seeds tested may be agamospermous (i.e. lacking an embryo), which has been observed in some *Rafflesia* species (Nais, 2001). Recently, Molina et al (2023) identified key genes in the *Rafflesia* seed transcriptome that may be involved in its germination, including genes for response to smoke-related chemical karrikin, as well as genes for laccase enzymes that in the unrelated plant parasite *Striga,* breakdown host tissue to facilitate infection. Thus in the future, it may be worthwhile to incubate *Rafflesia* seeds in laccase and karrikin in the presence of the host to induce germination. In 2017, two uninfected host cuttings (*Testrastigma harmandii* & *Tetrastigma cf. magnum)* that were collected from the Philippines have successfully rooted and are now growing at the USBG. These plants have been used as rootstocks for *Rafflesia*-infected cuttings collected in January 2023. It can take at least a year to know if the graft is completely successful, when a *Rafflesia* bud emerges from the grafted scion.

Since members of Rafflesiaceae are only detectable by their flowers and completely hidden within host tissues before they flower, conservation monitoring of populations has been difficult. Fortunately, Osanthanunkul (2019) successfully detected the presence of *Sapria himalayana* at different sites by using the eDNA (environment DNA)-based monitoring method. The eDNA-based monitoring method uses the amplification of specific DNA fragments that organisms release into the environment. Researchers have applied this monitoring method to aquatic animals, amphibians, and even mammals (Osathanunkul, 2019). Species like *Sapria himalayana* have low chances of survival due to their high bud mortality rate (46-67%) and low fruiting rate (8-12%). The study of Osathanunkul (2019) brought to light a successful monitoring method that could be applied towards conservation monitoring of *Rafflesia* and *Rhizanthes.*

## PLANT MICROBIOMES AND ENDOPHYTES

Given the difficulty in propagating members of Rafflesiaceae outside of their natural habitat using conventional horticultural methods, we have been exploring the microbiome of *Rafflesia* and its host *Tetrastigma.* Microbes that reside within plant tissues, or microbial endophytes, play a major role in promoting the growth of plants by producing certain growth regulators and/or protecting their plant hosts from pathogens. Bacterial endophytes that reside in the rhizosphere can enter internal plant tissues through primary and lateral root hair cells, root wounds, and the stomata from leaves and young stems (Santoyo et al., 2016). Proteobacteria is the dominant phylum that is generally found as bacterial endophytes. The two classes that are also found as endophytes are Firmicutes and Actinobacteria. Regarding the genera level, *Pseudomonas*, *Bacillus*, *Burkholderia*, *Strenotrophomonas*, *Micrococcus*, *Pantoea*, and *Microbacterium* are often found as endophytes (Santoyo et al., 2016). These bacterial endophytes have been demonstrated to produce hormones that promote plant growth and protect their plant hosts from pathogens and stressful environmental conditions (Santoyo et al., 2016).

Researchers have started to explore the positive impacts of bacterial endophytes and how they can be applied to improve agriculture. In a study, the endophytic community of sugarcane was investigated and researchers discovered that two strains, *Enterobacter and Shinella* were the true endophytes of commercial sugarcane as they were able to show plant growth promoting effects (Taulé et al., 2016). It is possible that there are unique microbial endophytes that may facilitate *Rafflesia* infection and can serve as bioinoculants to help *Rafflesia* grow ex situ. Like *Rafflesia, Phelipanche aegyptiaca* is a plant parasite which infects tomatoes, *Solanum lycopersicum.* Kruh et al (2017) studied the endophytic communities in non-parasitized tomato roots and parasitized tomato roots and found that when parasitized, the parasite and host had similar endophytic composition compared to the non-parasitized tomato root. This clearly indicates that the connection between the parasitic weed and the tomato host plant serves as a passageway for bacterial endophytes. Moreover, the researchers identified a new *Pseudomonas* sp. and found that it was able to reduce germination of the parasite seeds by 80% without harming the tomato host roots (Kruh et al., 2017). Their findings on the *Pseudomonas* strain could foster the use of biocontrol agents in controlling parasitic weeds, which could replace harmful chemical pesticides that are used in agriculture.

In the research study by Felestrino et al (2017), bacterial endophytes were also studied in the holoparasitic plant, *Langsdorffia hypogaea*, its various hosts, as well as from the soil community surrounding them, i.e the rhizosphere. This study led to the discovery of 81 bacterial isolates. 95% of the isolates were capable of fixing nitrogen and this gave the parasitic plants the ability to convert atmospheric nitrogen for their growth when soils were leached or contaminated with heavy metals. 86% of the isolates produced indoleacetic acid (IAA), which is a hormone that increases root growth and 20% of the isolates inhibited the growth of plant pathogens. This research sheds light on the importance of bacterial endophytes even to plant parasites. Thus, investigation of the bacterial endophytes within members of Rafflesiaceae could reveal how their microbiome could possibly benefit the growth and development of Rafflesiaceae for propagation and conservation.

In this study, the goal is to characterize the associated microbiome of Rafflesiaceae, particularly *Rafflesia speciosa* and *Sapria himalayana* to find "microbial markers" of Rafflesiaceae infection. It is hypothesized that these microbial markers will be present in Rafflesiaceae and their infected host plants but not in uninfected host plants. This study could also reveal "core" endophytic bacteria common in different species of Rafflesiaceae which may serve as bioinoculants to facilitate Rafflesiaceae propagation and conservation.

## METHODOLOGY

One of us, JM, collected various samples for microbiome characterization: eight (8) samples of host roots infected with *Rafflesia speciosa,* nine (9) *Rafflesia speciosa* buds, five (5) uninfected samples of host roots, and seven (7) samples of nonhosts (*Tetrastigma* spp. never observed to be parasitized by *Rafflesia*) were collected from Miagao, Iloilo, Philippines. In addition, two (2) buds of *Sapria himalayana* were collected

from Chiang Mai, Thailand. All collections were conducted under the appropriate permits and authorizations.

All samples were surface-sterilized with 2% sodium hypochlorite, rinsed, and processed for 16s microbiome sequencing (Illumina) and metagenomic analyses by Zymo Research (Irvine, CA). To deplete plant 16s rDNA chloroplast reads, sequence-specific peptide nucleic acid (PNA) blockers were used by Zymo. In addition, nanopore metagenomic sequencing was performed on seven *Rafflesia* flower buds to characterize their endophytic bacterial composition. The 16S Barcoding kit (SQK-16S024) was used for library preparation, and sequencing was performed on the MinION Mk1b device (Oxford Nanopore). The EPI2ME 16S-BLAST workflow was used for data processing to identify bacterial endophytes at the genus level.

Bacterial composition and proportions from Zymo's metagenomic analyses were used to make bar charts (Fig. 1-3) to better understand microbiome patterns. EPI2ME analysis of the nanopore reads for the *Rafflesia* buds produced a phylogeny of the bacterial endophytes (Fig. 4). Ecological characteristics of noteworthy bacterial taxa (Table 1) were investigated in the literature (Table 2) to gain insights on how these bacterial endophytes possibly facilitate infection and development of Rafflesiaceae.

## RESULTS

Figures 1-3 are bar charts showing bacterial proportions and compositions at phylum, class, and genus level. M-Raffbud, M-Infec, M-Uninfec, and M-Nonhost are *Rafflesia* speciosa-associated samples from Miagao (M), Philippines and T-Sapbud represents the *Sapria* himalayana buds from Chiang Mai, Thailand (T). Figure 4 shows the phylogeny of seven *Rafflesia* buds. Table 1 represents the different bacterial genera that were shared across the samples and Table 2 describes the ecological roles of these genera from the literature.

## DISCUSSION

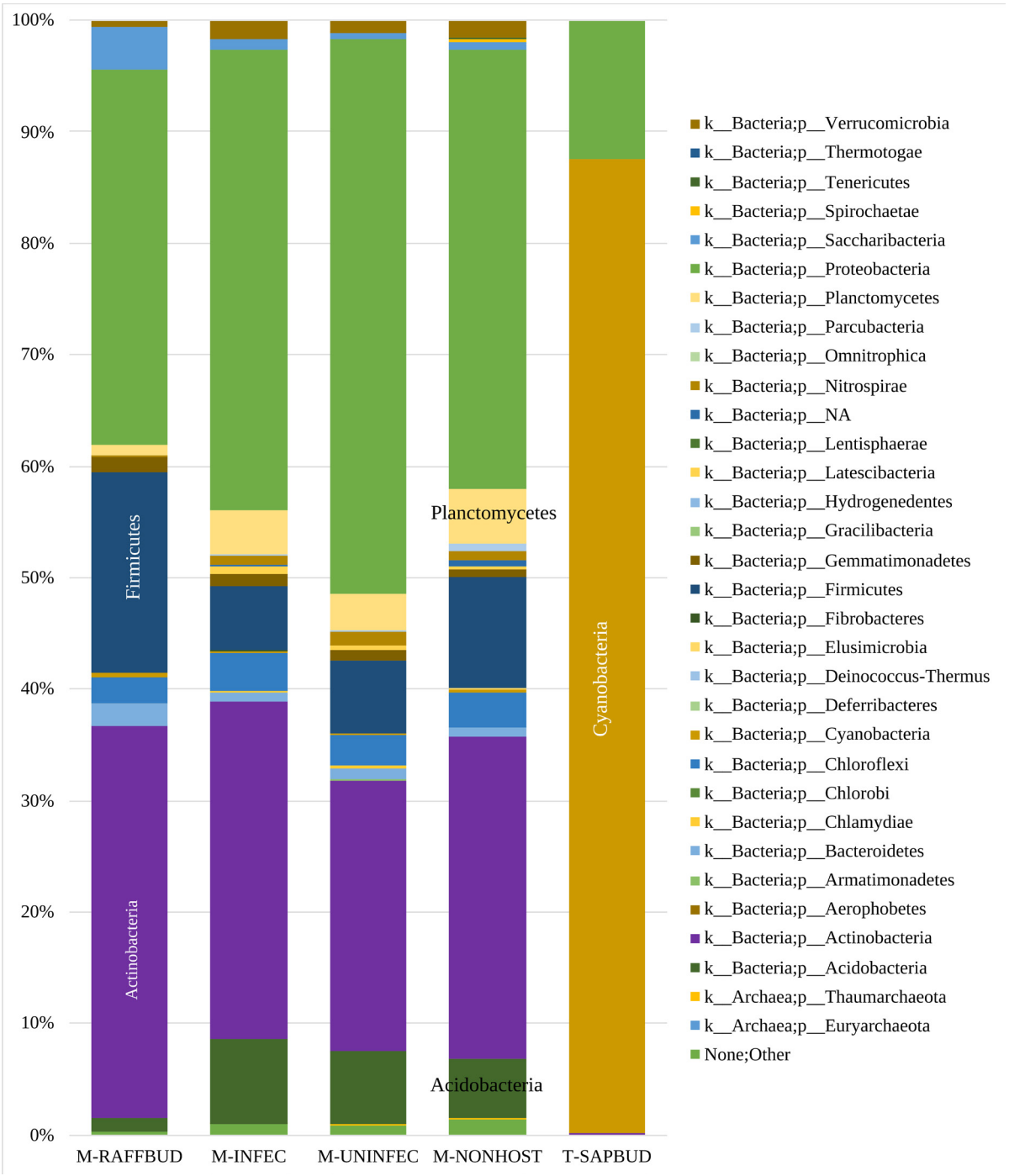
In this study, the goal was to characterize the associated microbiome of Rafflesiaceae, particularly *Rafflesia speciosa* and *Sapria himalayana* to find "microbial markers" of Rafflesiaceae infection, which may have growth-promoting properties that can facilitate Rafflesiaceae propagation and conservation. It was also hypothesized that *Rafflesia* and its host will have similar microbiomes given their intimate connection, similar to what was observed in *Phelipanche* and its tomato host (Kruh et al., 2017). However, results across different taxonomic levels (phylum Fig. 1; class Fig. 2; genus Fig. 3) demonstrate that *Rafflesia* buds have a distinct microbial endophyte composition and possessed some unique genera (Table 1) compared to its infected hosts. The *Tetrastigma* samples, whether nonhost or host, infected or not, visually have similar microbial composition and proportion especially at the Phylum and Class levels (Fig. 1-2). Interestingly, *Rafflesia* and *Sapria* differed dramatically in their microbiomes with *Rafflesia* having much more generic diversity (Fig. 3).

In Fig. 1, *Rafflesia* bud (M-Raffbud) has a slightly different phylum proportion from the *Tetrastigma* roots (M-Infec, M-Uninfec, and M-Nonhost) as it is enriched in Firmicutes, Saccharibacteria, and Actinobacteria, but it is diminished in Planctomycetes and Acidobacteria. Firmicute species are commonly known to have beneficial traits in plant growth promotion and agriculture (Hashmi et al., 2020). In two studies, Saccharibacteria played a major role in bacterial wilt disease suppression (Shen et al., 2018; Zhang et al., 2017). Similarly, Actinobacteria has a disease suppression ability because they are producers of secondary metabolites, which are products synthesized by bacteria that help with survival functions (Hazarika et al., 2020). Firmicutes, Saccharibacteria, and Actinobacteria could possibly provide plant growth promoting properties and protection against pathogenic bacteria to the *Rafflesia* bud as it transitions into a flower. The seemingly very large proportion of phylum Cyanobacteria in *Sapria* bud compared to *Rafflesia* bud, is probably an artifact, most likely from 16s rDNA chloroplast reads that were not affected by the PNA blockers used by Zymo. PNA clamps/blockers suppress plant host chloroplast DNA amplification and prevent host plant contamination to better analyze the bacterial communities in plants (Fitzpatrick et al., 2018). The large proportion of putative chloroplast reads in *Sapria* bud is interesting because Cai et al (2021) reported that *Sapria himalayana* lost its entire chloroplast genome, but the resulting data here suggest otherwise, or at least a remnant 16s rDNA has been retained by *Sapria*.

In Fig. 2, at the Class level, the bacterial composition of *Rafflesia* bud varies as well from the rest of the samples since it has a larger proportion of Clostridia and Coriobacteriia but a diminished proportion of Deltaproteobacteria and Acidobacteria. Clostridia has been shown to be a nitrogen-fixing endophyte present in the grass *Miscanthus sinensis* (Miyamoto et al., 2004). There is not much known about the plant growth promoting traits of Coriobacteriia but Coriobacteriia belongs to the phylum Actinobacteria, well known for enhancing plant growth through direct actions such as nitrogen fixation, phytohormones production, and siderophores production (Boukhatem et al., 2022). These effects could be potentially found in Coriobacteriia and may offer similar plant growth

## FIGURE 1

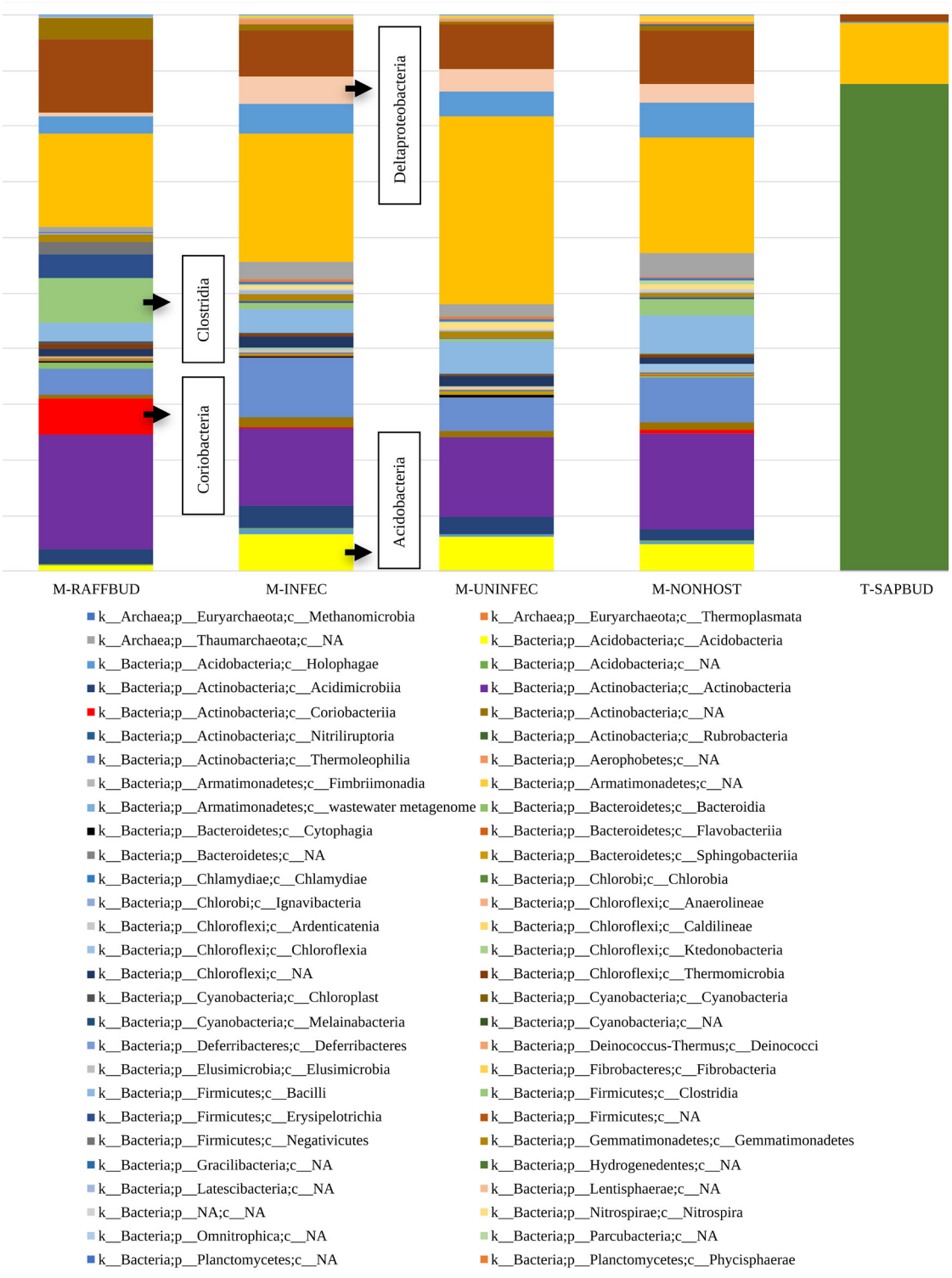
*Endophytic Bacterial Proportions and Compositions at Phylum Level of Rafflesiaceae samples from Illumina Sequencing*




Miagao *Tetrastigma* roots (M-Infec, M-Uninfec, and M-Nonhost) seem to have similar bacterial compositions and proportions regardless of Rafflesia infection, but M-Raffbud has a slightly different bacterial proportion from the rest of the samples with more Actinobacteria (purple) and Firmicutes (dark blue), yet diminished in Planctomycetes (yellow) and Acidobacteria (dark green). The bacterial compositions of T-Sapbud and M-Raffbud significantly differ from each other. Cyanobacteria largely composed the Sapbud microbiome (>87%, light brown). On the other hand, M-Raffbud only contains 0.34% of Cyanobacteria but has a higher microbial diversity than T-Sapbud.

## FIGURE 2.

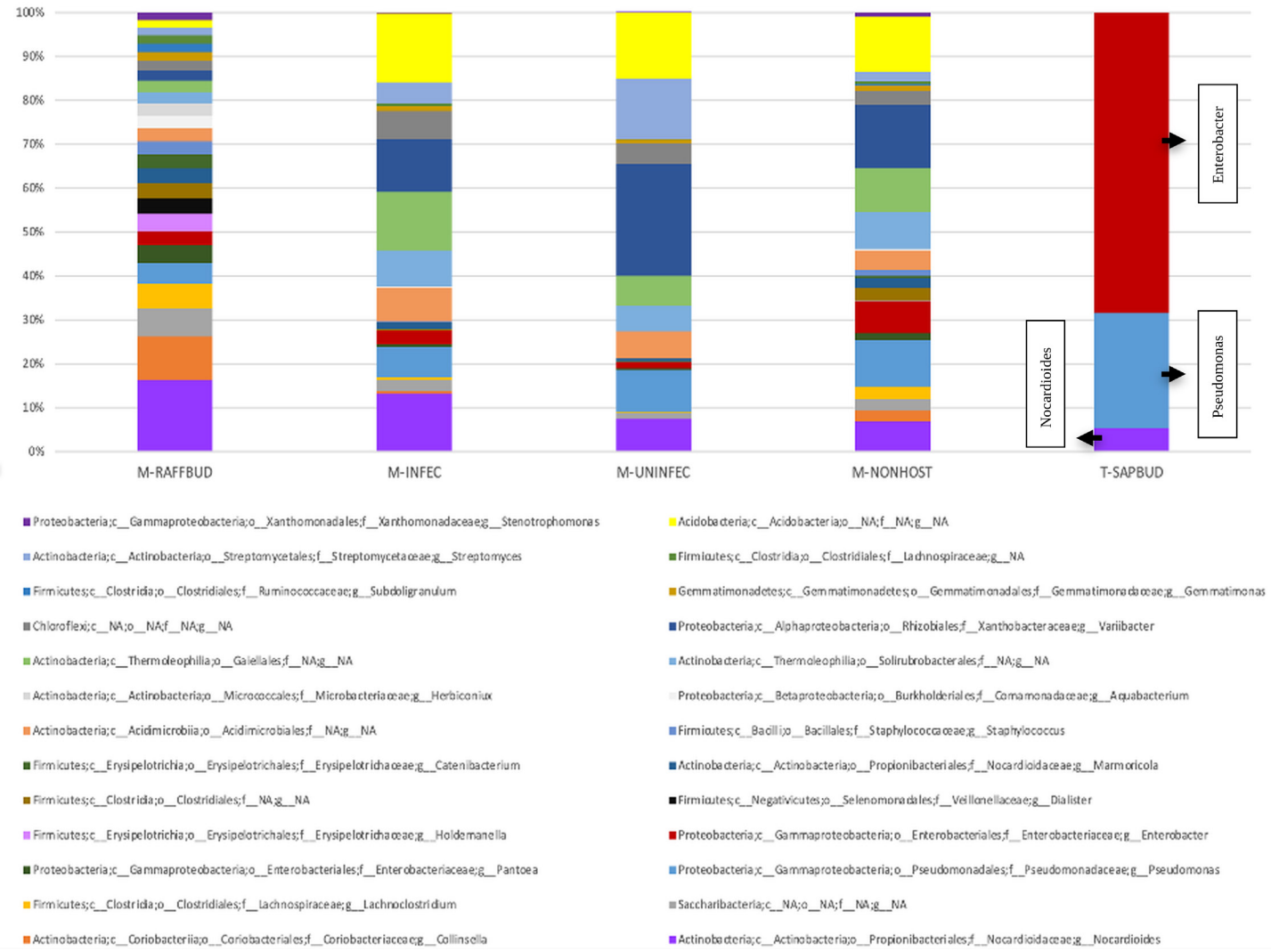
*Endophytic Bacterial Proportions and Compositions at Class Level of Rafflesiaceae samples from Illumina sequencing.*




*Rafflesia* buds (M-Raffbud) show bigger proportion of Clostridia (light green), Coriobacteria (red) but diminished proportion of Deltaproteobacteria (beige) and Acidobacteria (yellow) compared to the Miagao *Tetrastigma* roots, whether infected or not. The *Tetrastigma* roots (M-Infec, M-Uninfec, and M-Nonhost) seem to share similar bacterial compositions. The bacterial composition of T-Sapbud is significantly different from M-Raffbud.

## FIGURE 3.

*Endophytic Bacterial Proportions and Compositions at Genus Level of Rafflesiaceae samples from Illumina sequencing.*




This is the top 0.1% genera in *Rafflesia* (M-Raffbud) and *Sapria* (T-Sapbud) buds, which differ dramatically from each other in bacterial genera composition and proportions. *Sapria* bud only had 3 genera: *Enterobacter, Nocardioides, Pseudomonas* which were all present in the other samples as well. M-Raffbud also shows a different composition compared to the *Tetrastigma* samples, infected or not. *Herbiconiux* was among the top 0.1% unique genera in *Rafflesia* bud. Other unique M-Raffbud taxa are listed in Table 1.

## TABLE 1.

*Endophytic bacterial genera shared across samples from Ilumina sequencing. (RB=Raffbud, SB=Sapbud, TH=*Tetrastigma *host, NH=*Tetrastigma *nonhost)*

| Genus | Unique to RB | Present in both RB/SB | Unique to RB/TH | Unique to NH |
|---|---|---|---|---|
| *Holdemanella* (P. Firmicutes) | | | | |
| *Dialister* (P. Firmicutes) | | | | |
| *Herbiconiux* (P. Actinobacteria) | | | | |
| *Prevotella* (P. Bacteroidetes) | | | | |
| *Olsenella* (P. Actinobacteria) | | | | |
| *Bifidobacterium* (P. Actinobacteria) | | | | |
| *Propionibacterium* (P. Actinobacteria) | | | | |
| *Salinibacterium* (P. Actinobacteria) | | | | |
| *Buttiauxella* or *Lelliottia* (P. Proteobacteria) | | | | |
| *Pseudomonas* (P. Proteobacteria) | | | | |
| *Enterobacter* (P. Proteobacteria) | | | | |
| *Kluyvera* (P. Proteobacteria) | | | | |
| *Rhizobium* (P. Proteobacteria) | | | | |
| *Novosphingobium* (P. Proteobacteria) | | | | |
| *Klebsiella* (P. Proteobacteria) | | | | |
| *Pantoea* (P. Proteobacteria) | | | | |
| *Erwinia* (P. Proteobacteria) | | | | |
| *Flavobacterium* (P. Bacteroidetes) | | | | |
| *Kosakonia* (P. Proteobacteria) | | | | |
| *Mucilaginibacter* (P. Bacteroidetes) | | | | |
| *Shinella* (P. Proteobacteria) | | | | |
| *Rathayibacter* (P. Actinobacteria) | | | | |
| *Lactovum* (P. Firmicutes) | | | | |
| Arthrobacter or Glutamicibacter (P. Actinobacteria) | | | | |

## TABLE 2.

*Ecological characteristics of bacterial genera from Table 1.*

| Genus | Ecology of the bacteria | References |
|---|---|---|
| *Holdemanella (RB)* | This is an endophyte composing the microbiome of bamboo shoots but its ecological role is unclear. | Cui et al., 2021 |
| *Dialister (RB)* | This is an endophyte found in watermelon seed embryos but its ecological role is unclear. | Thomas et al., 2021 |
| *Herbiconiux (RB)* | This is an endophyte found in yerba mate (*Ilex paraguariensis*) and was positive for nitrogen fixation. Amidase, endoglucanase, phosphatase, and superoxidative dismutase were retrieved and classified as gene-clusters with plant growth-promotion (PGP) functions such as IAA-production, cellulose-degradation and anti-oxidant effects. | Pérez et al., 2016 |
| *Prevotella (RB)* | This is one of the bacterial genera found in the leaves of *Eucalyptus tereticornis* but ecological role is unclear. | Dasgupta et al., 2020 |
| *Olsenella (RB)* | No ecological information found in the literature. | N/A |
| *Bifidobacterium (RB)* | The strain *Bifidobacterium longum subsp. longum* is associated with metabolizing plant-derived poly/oligosaccharides, hemicellulose, and pectin-associated glycans. | Kelly et al., 2021 |
| *Propionibacterium (RB)* | This is an endophyte found in seeds of *Crotalaria pumila* growing on metal sites in Mexico but ecological role is unclear. | Sánchez-López et al., 2018 |
| *Salinibacterium (RB)* | *Salinibacterium* was found in *Stevia* leaf and its relative abundance was positively correlated with plant height. | Yu et al., 2015 |
| *Buttiauxella or Lelliottia (RB)* | The endophytic bacterium, *Buttiauxella* sp. SaSR13 was able to improve the growth of *Sedum alfredii* by 39 and 42% for shoot and root biomass. | Wu et al., 2018 |
| *Pseudomonas (RB/SB)* | Researchers investigated the effect of the nitrogen-fixing strain, *Pseudomonas stutzeri* A15, and discovered that A15 promoted the growth of rice seedlings. In another research study, the strain, *Pseudomonas brassicacearum* CDVBN10 was selected as a promising candidate to biofertilize rapeseed crops. | Pham et al., 2017; Jiménez-Gómez et al., 2020 |
| *Enterobacter (RB/SB)* | *Enterobacter* spp. are known to have many plant growth promoting characteristics that involve nitrogen fixation, soil phosphorus solubilisation, production of antibiotics, ability to secrete siderophores, etc. | Jha et al., 2011 |
| *Kluyvera (RB/SB)* | The bacterial strain EN4 (*Kluyvera ascorbata*) was isolated from cabbage and it was able to reduce *Plutella xylostella*. | Laurentis et al., 2014 |
| *Rhizobium (RB/SB)* | *Rhizobium* is a major contributor to nitrogen fixation, especially in leguminous plants. Researchers discovered *Rhizobium* species living in the stems of poplar trees, allowing them to grow in nutrient-poor soil. | Zahran, 1999; Doty et al., 2005 |
| *Novosphingobium (RB/SB)* | *Novosphingobium* sp. PS5 was selected for its plant growth-promoting potential in optimizing rice cultivation. | Rangjaroen et al., 2017 |
| *Klebsiella (RB/SB)* | The nitrogen-fixing *Klebsiella variicola* strain DX120E was shown to promote sugarcane growth. | Lin et al., 2015 |

*Table 2 continues on page 13*

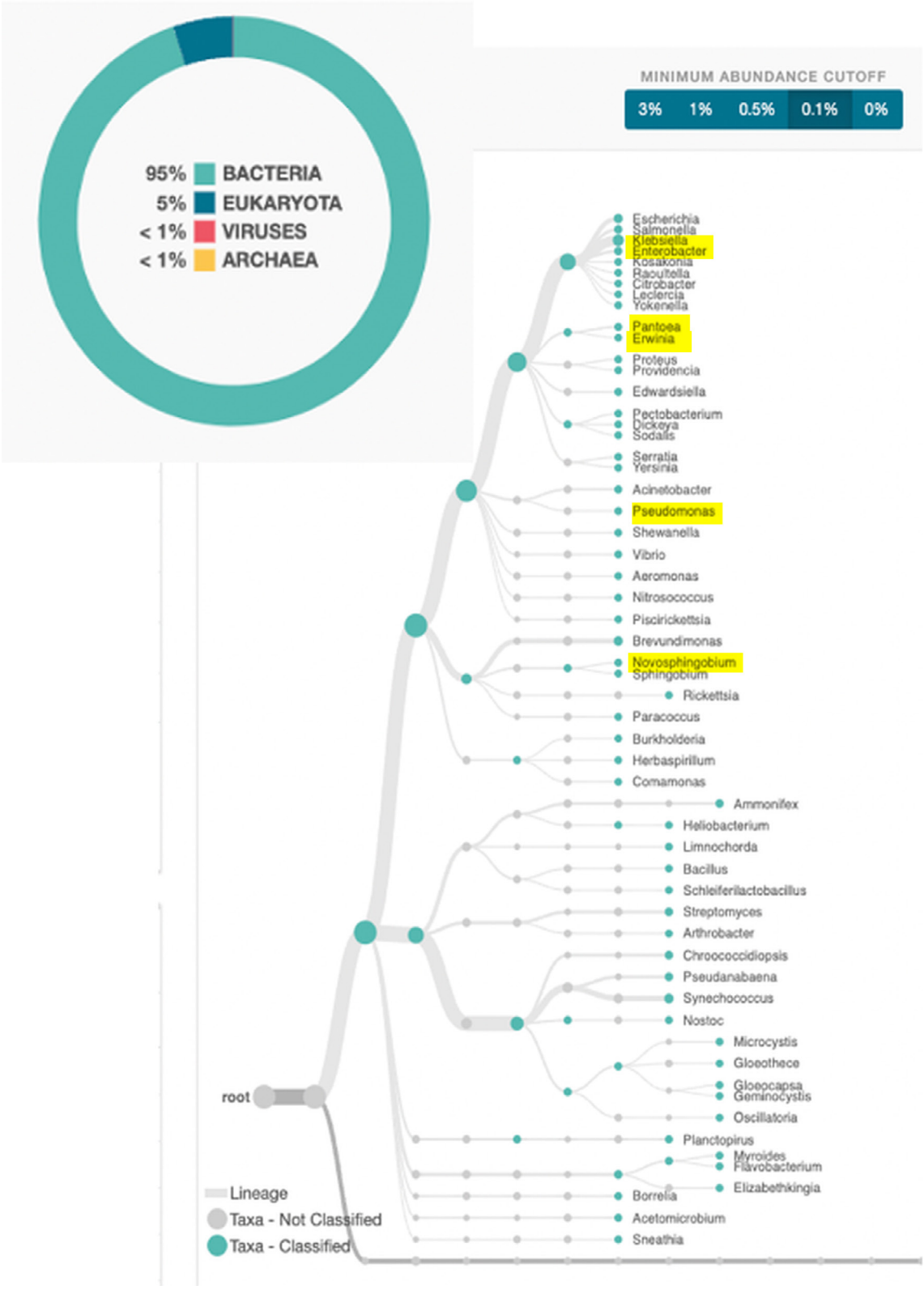
*Table 2 continued from page 12*

| Genus | Ecology of the bacteria | References |
|---|---|---|
| *Pantoea (RB/SB)* | In a study, *Pantoea agglomerans* MK-29 significantly reduced *Meloidogyne incognita* nematode infestation on tomato roots. | Feng et al., 2006 |
| *Erwinia (RB/SB)* | The endophytic bacterium, *Erwinia gerundensis* strain (A4) was tested for plant growth-promoting ability and the results showed that A4 had a positive effect growth on *Arabidopsis thaliana* as it increased its root hair length. | Guzmán et al., 2021 |
| *Flavobacterium (RB/TH)* | The genus *Flavobacterium* harbors many extra-cellular macromolecular-degrading enzymes. Some strains have been found to possess H2-oxidizing and ACC (1-aminocyclopropane-1-carboxylate) deaminase activities, which have been correlated to plant growth promotion. | Kolton et al., 2016 |
| *Kosakonia (RB/TH)* | *Kosakonia* sp. UYSO10 was able to promote sugarcane growth. | Taulé et al., 2019 |
| *Mucilaginibacter (RB/TH)* | *Mucilaginibacter* is known to have many roles in plant growth promotion. In a study, *Mucilaginibacter* was shown to alleviate salt stress in the plant model, *Arabidopsis thaliana.* In addition, they are bacteria that are capable of degrading pectin, xylan, laminarin and some other polysaccharides. | Fan et al., 2020; Broccanello et al., 2022; Pankratov et al., 2007 |
| *Shinella (RB/TH)* | In 2012, *Shinella* sp.UYSO24 was identified as a potential endophytic diazotroph for sugarcane (*Saccharum officinarum L.*) In 2016, the researchers identified strain *Shinella* sp.UYSO24 as a true endophyte of sugarcane plants. | Taulé et al., 2012; Taulé et al., 2016 |
| *Rathayibacter (RB/TH)* | In a study, researchers observed plant growth for pine seedlings that were inoculated with *Rathayibacter tanaceti* AN2r. | Padda et al., 2019 |
| *Lactovum (NH)* | Cells of the genus *Lactovum* are chemoorganotrophic and characterized by an aerotolerant anaerobic metabolism that yields lactate. | Drake et al., 2014 |
| *Arthrobacter or Glutamicibacter (NH)* | *Arthrobacter* was isolated from tomato rhizosphere and demonstrated plant growth promoting activities such as indole acetic acid (IAA) production. *Glutamicibacter* sp. FBE-19 was isolated based on its strong antagonism to the cucurbit bacterial blight pathogen *Erwinia tracheiphila* on plates. Members of the *Glutamicibacter* genus can promote plant growth under saline conditions and antagonize fungi via chitinolytic activity. | Banerjee et al., 2010; Fu et al., 2021 |

(RB=Raffbud, SB=Sapbud, TH=*Tetrastigma* host, NH=*Tetrastigma* nonhost). If there are two bacterial genera in the column, the taxonomic identity was ambiguous based on the database.

## FIGURE 4.

*Phylogeny of bacterial endophytes in Rafflesia buds.*




This phylogeny accounts for the top 0.1% in 7 *Rafflesia* buds with a total of 1,792,958 reads from Nanopore. The highlighted genera are also found in results from Illumina sequencing (cf. Table 1).

promoting benefits to *Rafflesia* bud.

At the genus level (Fig. 3), *Rafflesia* bud is quite different from the other samples in terms of microbial composition. It was hypothesized that *Rafflesia* buds will have a similar bacterial proportion and composition as its infected *Tetrastigma* hosts (M-Infec) but this was not the case with *Rafflesia* bud harboring some unique bacterial genera such as *Holdemanella, Dialister, Herbiconiux*, *Prevotella*, *Olsenella*, *Bifidobacterium*, *Propionibacterium*, *Salinibacterium* and *Buttiauxella-Lelliottia* (Table 1 and Table 2). There was no ecological role found for *Holdemanella* or *Olsenella* but *Holdemanella* is under phylum Firmicutes and as mentioned earlier, Firmicutes species are commonly known for having plant growth promoting traits (Hashmi et al., 2020). As for *Dialister*, there was no sufficient information on its plant growth promoting benefits but *Dialister* also belongs to phylum Firmicutes, and it was found as one of the abundant genera in watermelon seed embryos (Thomas et al., 2021). Many of the unique genera in *Rafflesia* bud that were found in the literature have plant-growth promoting properties such as nitrogen fixation and increase in plant biomass. In a research study, the endophyte *Herbiconiux* was positive for nitrogen fixation (Pérez et al., 2016). Another endophytic bacterium, *Buttiauxella* was able to improve the shoot and root biomass of *Sedum alfredii* by 39 and 42% respectively. There were unique genera in *Rafflesia* bud that were not detected in its associated host, which is surprising. It is possible that these were vertically transmitted via the *Rafflesia* seed when it germinated inside the host, and may be bacteria that do not readily cross between the host and parasite. These unique genera may be important to the germination of *Rafflesia* seeds and may facilitate propagation attempts from seed.

Excluding the very high proportion of chloroplast reads, *Sapria* bud had three genera having at least 0.1% proportion: *Enterobacter, Nocardioides, Pseudomonas,* which were also present in other samples *(Rafflesia* and *Tetrastigma)*. Even though *Sapria* bud only had three unique genera, they all demonstrated plant growth promoting benefits that involved production of antibiotics, plant growth under salinity stress, and plant growth on seedlings. *Enterobacter* species are known for their production of antibiotics, nitrogen-fixation ability, and secretion of siderophores. (Jha et al., 2011). In a study, the actinobacterium endophyte, *Nocardioides* sp. NIMME6 had many plant-growth promoting effects that allowed for the growth of wheat plants under salinity stress (Meena et al., 2020). In addition, the nitrogen-fixing strain, *Pseudomonas stutzeri* A15 promoted the growth of rice seedlings (Pham et al., 2017). These genera in *Sapria* bud may also serve as bioinoculants that can facilitate *Sapria* propagation.

There were other genera (not among the top 0.1%) shared by *Rafflesia* bud and *Sapria* bud such as *Kluyvera, Rhizobium, Novosphingobium, Klebsiella, Pantoea, and Erwinia* (Table 1, 2). These taxa were also found in the *Tetrastigma* samples so are not unique to Rafflesiaceae. The plant growth promoting traits of the genera that were shared in *Rafflesia* and *Sapria* bud included nitrogen fixation and plant growth promoting ability. For example, the genera *Rhizobium* is a major contributor to nitrogen fixation, especially to the stems of poplar trees, which are trees that have the ability to grow in nutrient-poor soil (Zahran, 1999; Doty et al., 2005). The nitrogen-fixing *Klebsiella variicola* strain DX120E was able to promote sugarcane growth (Lin et al., 2015). *Rhizobium* and *Klebsiella* are diazotrophic endophytes and they could both potentially provide nitrogen fixation to *Rafflesia* and *Sapria.* The endophytic bacterium, *Erwinia gerundensis* strain (A4) had a positive effect growth on *Arabidopsis thaliana* as it increased its root hair length (Guzmán et al., 2021). These genera were shared by *Rafflesia, Sapria,* and *Tetrastigma* samples. The shared genera between the samples could potentially be common endophytic taxa of plants.

Furthermore, *Rafflesia* buds and its *Tetrastigma* host also share genera, as expected given their intimate association. These shared genera include *Flavobacterium*, *Kosakonia*, *Shinella, Mucilaginibacter* and *Rathayibacter*. *Kosakonia* sp. and *Shinella* sp. were reported to promote sugarcane growth (Taulé et al., 2016; Taulé et al., 2019). *Mucilaginibacter* is known to have many roles in plant-growth promotion, and it was shown to alleviate salt stress in the plant model, *Arabidopsis thaliana* (Fan et al., 2020; Broccanello et al., 2022). *Flavobacterium* strains have been found to possess H2-oxidizing and 1-aminocyclopropane-1-carboxylate deaminase activities (ACC), which have been correlated with plant growth promotion (Maimaiti et al., 2007; Kolton et al., 2016). H2 plays a key role in plant development, with positive effects on seed germination as well as root elongation (Li et al., 2018). Bacteria that produce ACC deaminase activities can directly promote and protect plant growth (Glick, 2014)

Though the genus *Tetrastigma* is the sole host to Rafflesiaceae species, not all *Tetrastigma* species can serve as hosts. In fact, only 11 of 95 *Tetrastigma* species serve as *Rafflesia* hosts (Pelser et al., 2016). There may be unique microbiota in *Tetrastigma* non-host species that could be antagonistic to *Rafflesia* infection such as *Lactovum*, *Arthrobacter*, and *Glutamicibacter* which were identified in non-host species in this study. Cells of the genus *Lactovum* yield lactate; lactic acid bacteria strains have been considered promising candidates in agriculture for their ability to promote plant growth and show antagonistic effects against pathogens (Drake et al., 2014; Raman et al., 2022). *Arthrobacter* demonstrated plant

growth promoting activities such as indole acetic acid (IAA) production and *Glutamicibacter* was able to promote plant growth under saline conditions and antagonize the pathogen, *Erwinia tracheiphila* (Banerjee et al., 2010; Fu et al., 2021). Perhaps these unique bacteria somehow suppress *Rafflesia* infection conferring some immunity to the *Tetrastigma* non-host species.

The phylogeny of endophytic bacteria in *Rafflesia* bud from Nanopore in (Fig. 4) shows six genera that were also characterized by Zymo (Table 1). They are *Klebsiella*, *Enterobacter*, *Pantoea*, *Erwinia*, *Pseudomonas*, and *Novosphingobium*. However, there were also discrepancies between the two datasets when comparing the *Rafflesia* buds. In Zymo, there were 87 genera with at least 0.1% abundance, while in Nanopore sequencing, 57 genera were characterized with 0.1% abundance (Fig 4). An explanation to why Zymo and Nanopore have differences in genera could be because the *Rafflesia* buds samples were collected at different sites. Also, it is possible that Zymo and Nanopore used different 16S primers sets. An issue that arose with Nanopore sequencing was not having sufficient microbial DNA concentration even after experimenting with various extraction kits.

In conclusion, this research has provided insight into the microbiome of Rafflesiaceae species. Microbial taxa present in both *Rafflesia* and *Sapria* bud could be potentially used as bioinoculants in propagation attempts of these horticulturally recalcitrant taxa. The identification of unique bacterial taxa in *Rafflesia* buds despite its close association with its host is interesting and may have been vertically transferred via *Rafflesia* seeds and could thus be vital in stimulating *Rafflesia* seed germination in vitro or in planta. The identification of unique bacteria in *Tetrastigma* non-host species could suggest that there are endophytic microbes that could confer some immunity to non-host species and ecologically antagonize *Rafflesia* infection. This study unveils the symbiotic microbes of *Rafflesia* and *Sapria* and their potential horticultural application in propagating the world's largest flowers.

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# Characterization of Pigments consisting of Melanin and Carotenoids from a *Planococcus* Species

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## ABSTRACT

*Planococcus* sp. CAU13 produces a dark pigment, the identity of which is the focus of this study. Initial extraction methods utilized known procedures for carotenoid extraction, as *Planococcus* members are known to produce yellow to orange pigmented carotenoids. Extractions were followed by melanin isolation techniques, as the carotenoid methods yielded a yellow, but not the expected dark color. Ultraviolet–visible (UV-Vis) spectroscopy and infrared (IR) spectroscopy were used to characterize the extractions. Analysis found that both carotenoids and melanin are likely present. Genetic analysis supports the presence of both carotenoids and melanin. Portions of a known C-30 carotenoid biosynthetic gene cluster (BGC) are present in CAU13. which produces a type III polyketide synthase (PKSIII); this is a class of enzyme known to produce 1,3,6,8-tetrahydroxynaphthalene (T4HN), the first product in the biosynthetic pathway for DHN-type allomelanin. Multiple experiments verify the hypothesis that a mixture of a carotenoid and melanin are produced by the bacterium *Planococcus* sp. CAU13.

**Keywords**: *Planococcus*, Melanin, Carotenoid, Pigment, Extraction, CAU13, Polyketide Synthase

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## INTRODUCTION

*Planococcus* sp. CAU13 was first isolated and cultured from a soil sample extracted from the desert sands of Hashemite Kingdom of Jordan. Isolation revealed spherical smooth colonies presenting a dark pigment morphology after 10-20 days of growth (Fig 1). The bacterium's genome was sequenced and it was determined to be a member of the *Planococcus* genus (1). Other members in the genus are known to produce orange and yellow pigmentation due to the presence of carotenoids (2; 3). Carotenoids are a class of biomolecules produced by fungi, microorganisms, and plants (4). These biomolecules serve as pigments and give rise to orange, yellow, and red colors due to their absorbance in the visible spectrum via their conjugated system of single and double bonds, created by their origin as tetraterpene derivatives (5). Carotenoids can be divided into two types, xanthophylls and carotenes. Xanthophylls contain oxygen and are typically the type of carotenoids that *Planococcus* members produce, while carotenes are pure hydrocarbons. Microorganisms can produce a variety of carotenoids, for example *Arthrobacter* sp. NamB2 produces a pink pigment, the heterothallic zygomycetous fungus, *Blakeslea trispora* produces $\beta$-carotene, a yeast *Phaffia rhodozyma* (also known as *Xanthophyllomyces dendrorhous*) produces $\beta$-carotene and astaxanthin, bacteria such as *Croceibacterium* produces zeaxanthin, *Micrococcus luteus* produces yellow pigment (5)

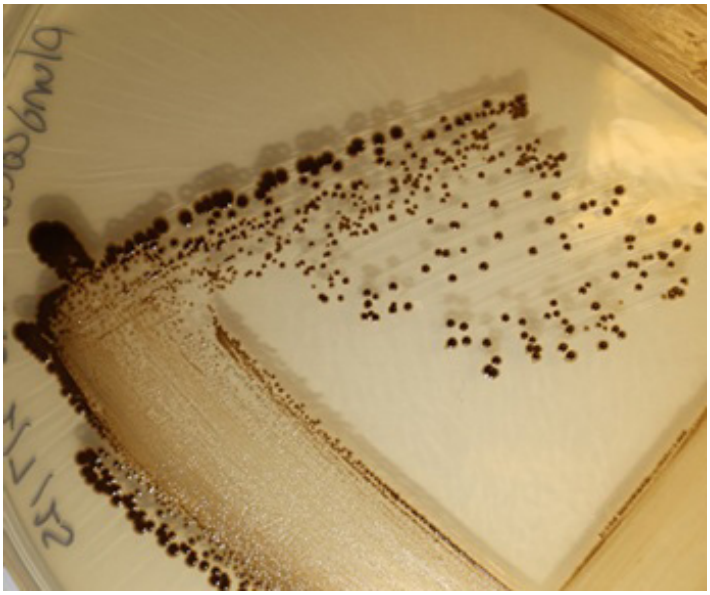
Carotenoids primarily have two functions, absorbing light energy to aid in photosynthesis, and providing photoprotection while acting as an antioxidant (3). Due to the seemingly conserved nature of carotenoids throughout the genus, methods for carotenoid extraction were utilized to determine whether they play a role in the pigmentation of the focus of this study *Planococcus* sp. CAU13. Previous unpublished studies from this laboratory have shown the possible presence of carotenoids, but have not yet elucidated the identity of the black pigment or investigated the genetic origin of carotenoid production.

While no other member of the genus is known to exhibit black pigmentation one member, *Planococcus maritimus*, produces 1,8-dihydroxynapthalene (DHN) which is a precursor to allomelanin (5). Melanin is another biomolecule which is ubiquitous in all forms of life responsible for a brown to black range of pigmentation. Melanin's primary function is for protection against environmental factors including DNA damage from UV radiation, high temperatures, and chemical stressors like oxidation. Allomelanin, the type that *P. maritimus* is hypothesized to produce, is most common in fungi (6) but is present in other bacteria (7; 8; 9; 10). Production of allomelanin through the DHN pathway involves the use of a class of enzyme called a type III polyketide synthase (PKSIII). PKSIII catalyzes the formation of (1,3,6,8)-tetrahydroxynapthalene (T4HN) utilizing malonyl-CoA as a substrate, which is followed by enzymatic reduction and dehydration steps to form 1,8-dihydroxynaphthalene (1,8-DHN) (7). PKSIII enzymes exist as homodimer proteins which produce a wide variety of products (11). A PKSIII involved in bacterial T4HN production as a melanin precursor has been described in both its structure and its active site which allows for the comparison to possible homologous enzymes in CAU13 (11).

The goal of this study is to isolate and characterize the black pigment and determine if *Planococcus* sp. CAU13 produces either or both melanin and carotenoids. As previous work suggested the presence of carotenoids, initial methods for preliminary extraction included established procedures for carotenoid extraction. This was followed by a procedure for melanin extraction, as carotenoid methods did not yield the desired black pigmentation. Both methods were followed by IR and UV-Vis spectroscopy to characterize the extract, and genetic analysis was performed to understand the pathways involved.

## FIGURE 1

Planococcus *sp. CAU13 grown on marine agar plate with 5% NaCl.*



Colonies exhibited dark brown glossy color.

## MATERIALS & METHODS

### CULTURE GROWTH

*Planococcus* sp. CAU13 was streaked onto plates of Zobell Marine Broth 2216 supplemented with 5% NaCl and 7.5% agar. Cultures were grown in the dark at room temperature (23 ℃) for two weeks until the dark pigment appeared. The dark pigmented colonies were scraped into 15 mL conical screw cap tubes for extraction of the pigment.

### CAROTENOID BASED EXTRACTION

Preliminary extraction utilizing carotenoid methods dissolved a collected plate of CAU13 in 2.0 mL of a 7:3 mixture of acetone: methanol containing 0.1% BHT as an antioxidant (3). The mixture was incubated at 50 °C for 50 minutes and sonicated for 15 seconds before being placed in a centrifuge at 11,000 xg rcf for 5 minutes and the supernatant was collected. Diethyl ether was used as replacement solvent in a secondary extraction following the same procedure to adjust for nonpolar factors and ease of residue collection.

### MELANIN BASED EXTRACTION

Preliminary extraction utilizing melanin methods (3; 13) followed the same method as carotenoid extraction (5; 12). A collected plate of CAU13 was washed with 2 mL of hexane then the remaining residue was dissolved in 2 mL of 2M NaOH (12). The mixture was incubated at 50°C for 50 minutes and sonicated for 15 seconds before being placed in a centrifuge at 11,000 xg for 5 minutes and the supernatant was collected.

### IR-SPECTROSCOPY

Carotenoid-based diethyl ether extracted supernatant was air dried in fume hood and the solid residue was collected. This residue was scanned directly on a JASCO FT/IR-4X1typeA IR spectrometer from 4000-500 $cm^{-1}$. Melanin extract was precipitated using 2M HCl dried and collected. This extract was scanned directly on a JASCO FT/IR-4X1typeA IR spectrometer from 4000-500 $cm^{-1}$.

### UV-VIS SPECTROSCOPY

The supernatant of the melanin and carotenoid extracts were scanned on a Perkin Elmer Lambda 35 UV-Vis spectrometer from 300-800 nm; melanin-based extract required a 40x dilution with 7:3 v/v acetone:methanol from the original concentration prior to analysis.

### PROTEOGENOMIC ANALYSIS

The online genomic database collection website BioCyc (14) was used for investigating the genome of CAU13 to identify hypothetical polyketide synthase. T-Coffee "Expresso" alignment from T-Coffee (15) was used for BLAST analysis comparing specific polyketide synthase in CAU13 with other organisms.

## RESULTS

### CAROTENOID EXTRACTION

Carotenoid extraction method that used 7:3 acetone:-methanol yielded a yellow-orange supernatant, Figure 2. UV-Vis full spectrum scan yielded three distinct peaks at 440.5, 462.6, and 484.1 nm, Figure 3. IR spectra exhibited bands at 3300 $cm^{-1}$, 1535 $cm^{-1}$, 1400 $cm^{-1}$, 1340 $cm^{-1}$ Figure 4.

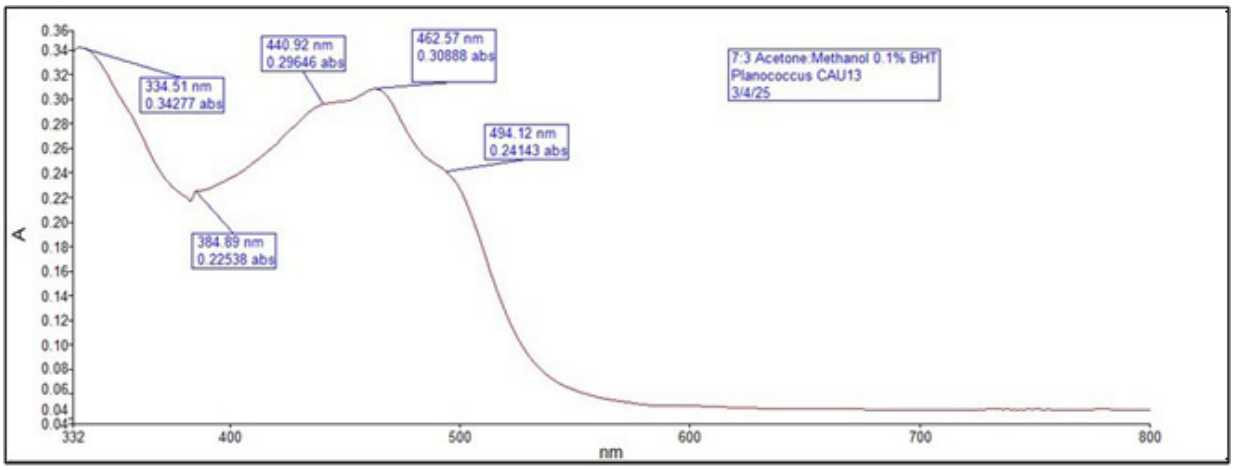
## FIGURE 2

*Carotenoid extraction with 7:3 v/v acetone:methanol.*

Color of the extract appears yellow to light brown.

## FIGURE 3

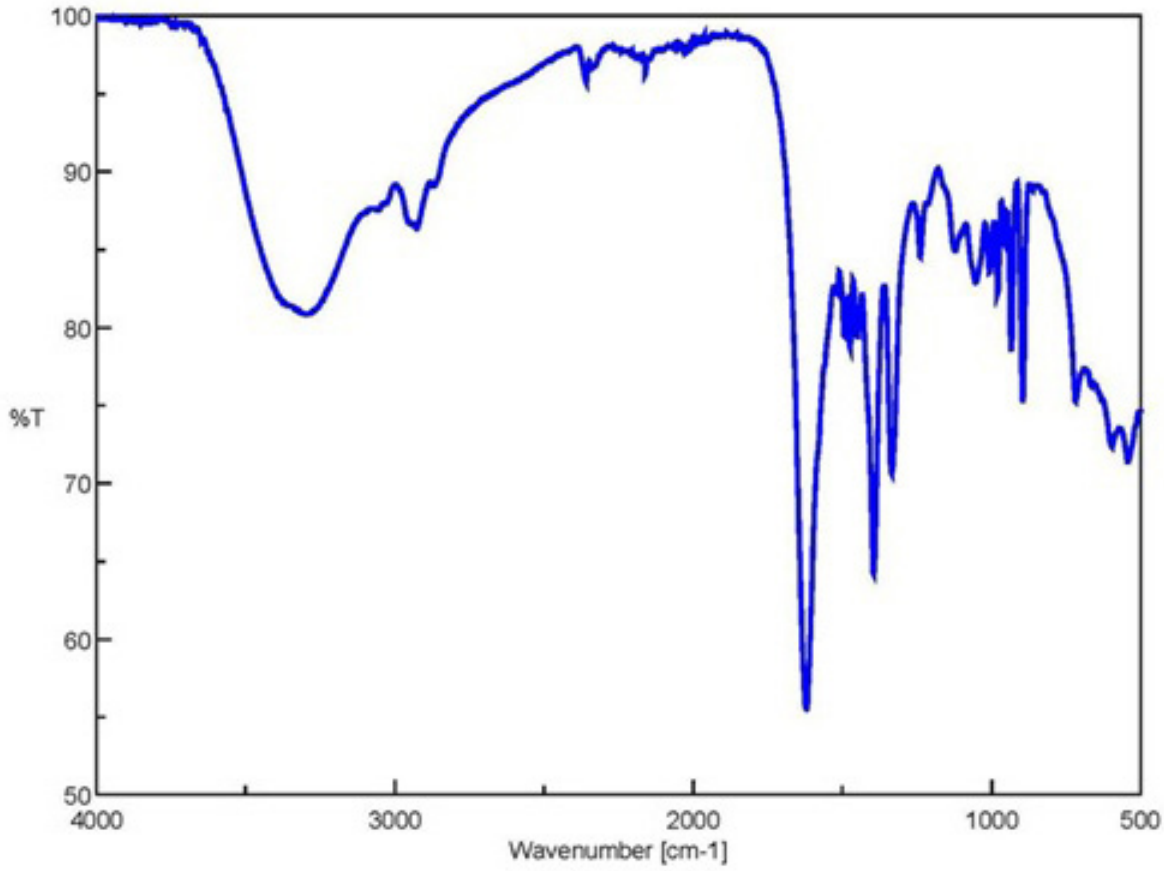
*UV-Vis spectrum of the carotenoid extraction with 7:3 v/v acetone:methanol.*




Prominent spectral features are 334 nm peak, 385 nm valley, 463 nm peak with 441 nm and 494 shoulders.

## FIGURE 4

*IR spectrum of the carotenoid extraction with 7:3 v/v acetone:methanol.*




Prominent spectral bands are broad absorbance between 3300 and 3500 $cm^{-1}$, and sharp bands at 1535 $cm^{-1}$, 1400 $cm^{-1}$, and 1340 $cm^{-1}$.

## MELANIN EXTRACTION

Melanin extraction methods using 2 M NaOH yielded a dark brown supernatant liquid, Figure 5. UV-Vis analysis of the liquid showed peaks and maximum at 223.31 nm and 244.88 nm, Figure 6. IR spectral analysis showed broad absorbance at 3300-3500 $cm^{-1}$, sharp bands at 1627 $cm^{-1}$, 1540 $cm^{-1}$, and 1051 $cm^{-1}$, Figure 7.

## FIGURE 5

*Melanin extraction using 2 M NaOH.*

Color of the extract appears brown.

## FIGURE 6

*UV-Vis spectrum of melanin extraction using 2 M NaOH.*

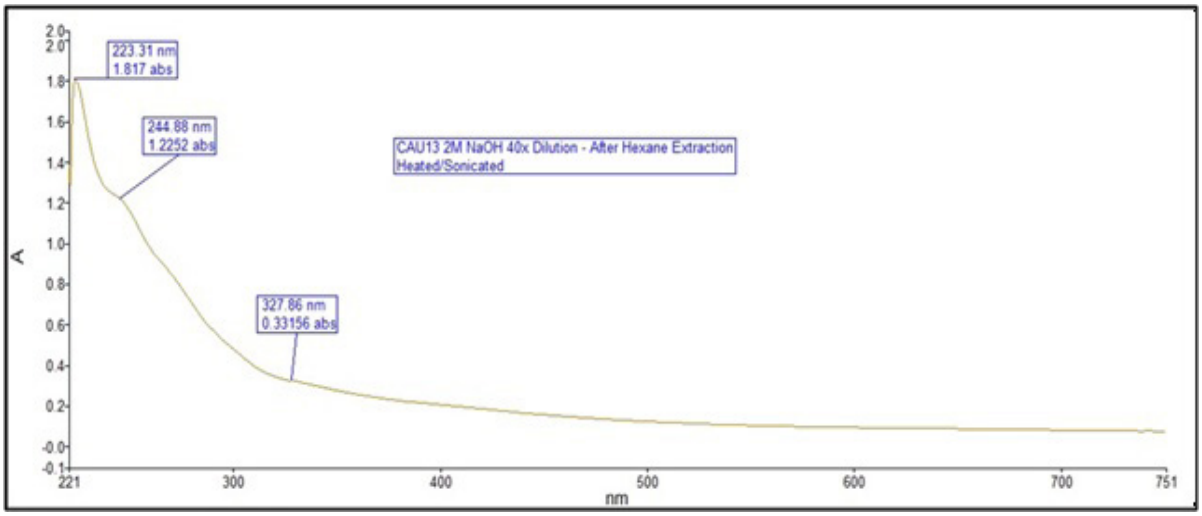
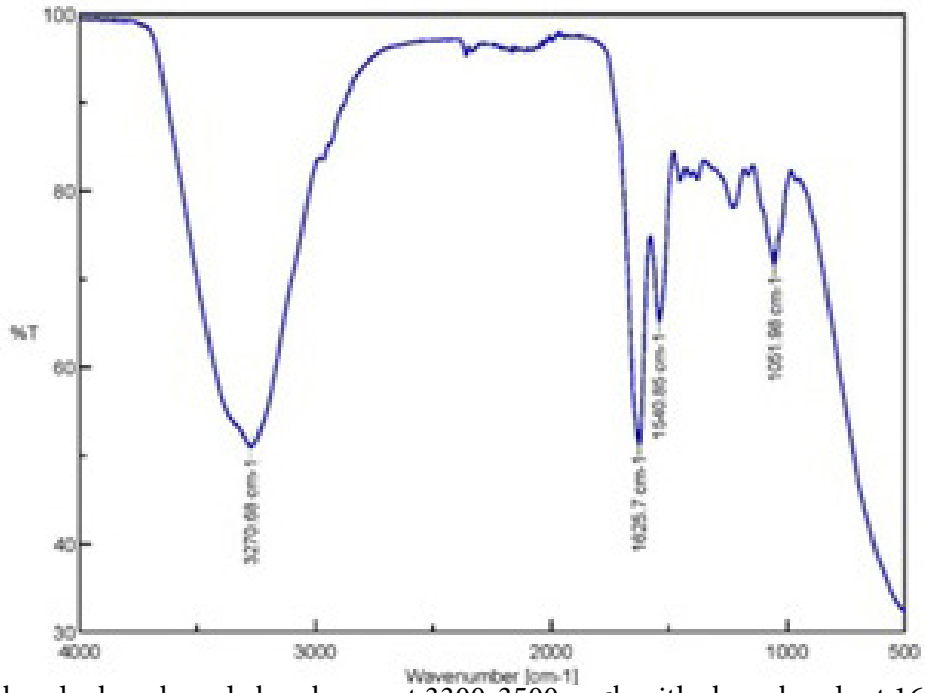



Figure 6: UV-Vis spectrum of melanin extraction using 2 M NaOH.
Prominent spectral features peaks at 223 nm with shoulders at 245 and 327 nm.

## FIGURE 7

*IR spectrum of melanin extraction using 2 M NaOH.*




Prominent spectral bands show broad absorbance at 3300-3500 $cm^{-1}$, with sharp bands at 1627 $cm^{-1}$, 1540 $cm^{-1}$, and 1051 $cm^{-1}$,

## DNA AND PROTEIN SEQUENCE ANALYSIS

### FIGURE 8

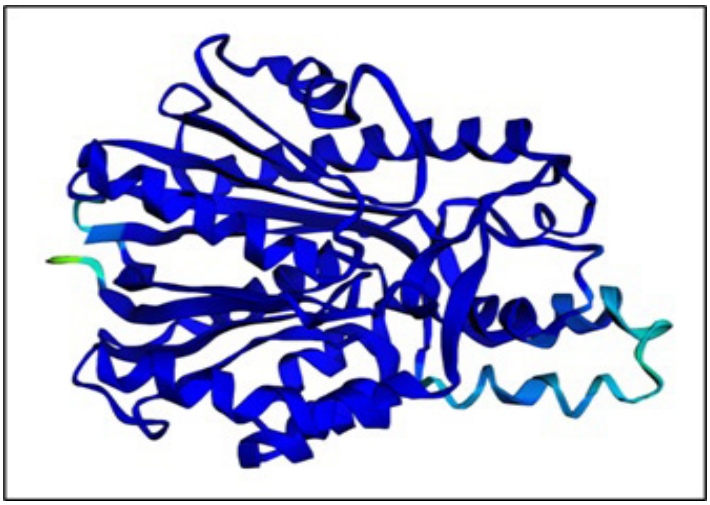
*Multiple sequence alignment using T-COFFEE (13).*

```
T-COFFEE, Version_11.00 (Version_11.00)
Cedric Notredame
SCORE=92
*
 BAD AVG GOOD
*
AUX21439.1        :  91
WP_033543849.1    :  93
WP_152152970.1    :  92
WP_394120825.1    :  93
WP_088007427.1    :  92
cons              :   9

AUX21439.1        MSTGHHEMPFAPMIAAVGTALPPHYATQEETCAGLRRLWAGKGDALALVERVQRSIEVRGRHLAMP
WP_033543849.1    M----------SIIRKVVTENAPYHVDQKEIVQVVRNLFGDHFSDIERLLRVFGNGQIEGRYFAAP
WP_152152970.1    M----------PFIRSVVTENPPYRFDQQDIVEIVRNLFGGYYDDIERLLRVFGNGQIENRHFAAP
WP_394120825.1    M----------SMIRKVVTENAPYYVDQKEIVSVVRNLFGGHYTDIERLLKVFGNGQIEGRYFAAP
WP_088007427.1    M----------PYIRNVATANAPYKVDQQQTVEVVRNLFGSQFADIERLLKVFDNGEIESRYFAAP

cons              *          . *  * *  .*:   *::   :*.*:..    :  : :*  . :::.*::* *

AUX21439.1        VEAYHAASTFAEQNRRWNACALELGERACRAALERAGLLPEDIDQIVFVSVTGISAPSLDARLVNR
WP_033543849.1    LEWFEKERGLEEKNRLYIEEAVRMGSRAVERCLEESGTAKEEVDAFIFVSSSGMATPTIDARIMNN
WP_152152970.1    LSWFEKEHGLEEKNQKYIEEAVRLGSRAVANCLKKADVNKEEIDAFVFVSSSGMATPTIDVRIMNE
WP_394120825.1    LVWFEEERGLEEKNRLYVEEAVKMGSLAVSRCLEEAQVDKEDVDAFIFVSSSGMSTPTIDARIMNV
WP_088007427.1    LDWFEQERGLEEKNGRYVDQAVEMGSDAVMRSLAKARLSPDDIDAFIFVSSSGMATPTIDARIMNK

cons              :  :.   : *:*  :   *:.:*. *   .* .:    :::* ::*** :*:::*::*.*::*

AUX21439.1        LGFRRDVKRTPIFGLGCVAGVAGVARVTDTLRAFPAHTSLLLSVELCSLTLQRDDTSAANVIAAQL
WP_033543849.1    LKMPAHIKRLPIWGLGCAGGAAGLSRAHDYCRAYPDAKVLVLCLELCSLTFQRSDLTKSNLIGTSL
WP_152152970.1    LSLPSHIKRMPLWGLGCAGGAAGISRAHDYCLAYPNAKVLVLCIELCSLTFQRSDTSKSNLIGTSL
WP_394120825.1    LQMPQHVKRIPLWGLGCAGGASGISRADDYCRAYPEAVVVVLCLELCSLTFQRSDTSKSNLIGTSL
WP_088007427.1    LAFPPHVKRIPLWGLGCAGGAAGISRAHDFCLAYPEATVLVLCIELCSLTFQRQDLSKSNLIGTSL

cons              * :  .:** *::****..*.:*::*. *   *:*    ::*.:******:**.* : :*:*.:.*

AUX21439.1        FADGAAAVVARGAQAARKSGRAGGAAVLATHAVFYPDTEHIMGWDVVDSGFRVVLSAGVPALVREH
WP_033543849.1    FADGSACALVTGERAA---GDDAGFFIHDTQSTLMPESEDVMGWDVKDEGLHVVFSRDIPKIIESW
WP_152152970.1    FADGAACALVTGEEGQ---TAGAGFFTKDTQSTLMSNSEDVMGWDVKDEGLHVIFSRDIPNIVESW
WP_394120825.1    FADGAACALVTGDSVR---LPGEGFHIKETQSTLMRDSENVMGWDVKDEGLHVVFSRDIPKIIEKW
WP_088007427.1    FADGAACALVSGDRTA---GAD-GFFIRDTQSTLMPESEEVMGWDVKDGGLHVVFSRDIPSIIENW

cons              ****:*..::. *      *    *::.:  ::*.:***** * *::*::* .:* ::..

AUX21439.1        VRRDVDAFLARHGLSRRDVRHWIVHTGGPKVLEAVAETLELPGDALDRSWRSLREIGNVSSASVLF
WP_033543849.1    LKPNLDQFLEKLDRKPEDIEHFIAHPGGKKVLSAYEKSLSIGTGKTENPRNVLRDFGNMSSPTVLY
WP_152152970.1    LKPNVDLFLDKLGKKSSEIDHFIAHPGGKKVLEAYEKSLGFSAEKTAVSRKVLASHGNMSSPTVLY
WP_394120825.1    LKPNVDLFLGGISRTSADITHFIAHPGGKKVLAAYEKSLGLATGKTAISRAVLAKYGNMSSPTVLY
WP_088007427.1    LKPNVDGFLDSIGKTGADIDHFIAHPGGKKVLAAYEKALDISREKTAVSREVLTRFGNMSSPTVLY

cons              :: ::* **   . .  :: *:*.*.** *** *  ::* :       .   *   **:**.:**:

AUX21439.1        VLRDFLDE-PCARPGDHGVMMAFGPGFCSEMALLRW------
WP_033543849.1    VLKEFMEQQPNA--GEQGLLTALGPGFSSEMLWLEWR-EKSG
WP_152152970.1    VLKEIIENQPAA--REQGLLTALGPGFSSEMLWLEWG-EM-E
WP_394120825.1    VLKDFMEKKPRQ--GEEGLLTALGPGFSSEMLWLEWG-ESVL
WP_088007427.1    VLKEFMEQEPAD--GTNGLLTALGPGFSSELVWLEWEGLM-E

cons              **:::::: *    .*:: *:****.**:  *.*
```

NCBI reference sequence numbers represent the protein sequence for the respective organism. AUX21439.1 chalcone synthase *Sorangium cellulosum*, WP_033543849.1 type III polyketide synthase *Planococcus* sp. CAU13, WP_152152970.1 type III polyketide synthase *Bacillus aerolatus*, WP_394120825.1 type III polyketide synthase *Planococcus donghaensis*, WP_088007427.1 type III polyketide synthase *Indiicoccus explosivorum*.

### FIGURE 9

*ColabFold v1.5.5: AlphaFold2 using MMseqs2 Structure Prediction of CAU13 PKSIII production from KW93 (14).*



## DISCUSSION

### CAROTENOID

The carotenoid extraction method using 7:3 acetone:-methanol yielded a yellow-orange supernatant (Figure 2) UV-Vis of which (Figure 3) provided results consistent with carotenoids, three distinct peaks are present at 440.52, 462.57, and 484.12 nm which are common for *Planococcus* xanthophylls (2; 15; 16). These three major maxima suggest a chromophore which is composed of nine conjugated double bonds, the typical length of xanthophylls (17). IR spectra shown in (Figure 4) after collection from diethyl ether is consistent with *Planococcus* carotenoids with absorption at labeled values described in previous studies (18). The broad absorbance at ~3300 $cm^{-1}$ suggests the presence of hydroxyl groups which supports the xanthophyll identity of carotenoid(s) produced by CAU13. IR spectra obtained for acetone:methanol mixture provided inconclusive results, improved procedure for solvent removal may provide a higher quality spectrum. As diethyl ether is a nonpolar solvent in comparison to the acetone:-methanol mixture, analyzed extract may include a higher ratio of nonpolar carotenoid precursor molecules. Both spectroscopic techniques therefore support the previous conclusion that carotenoids are present in CAU13 and reasonably would be responsible for a yellow/orange pigmentation.

IR spectra provide overlapping results that match both carotenoids and melanin within the fingerprint region, <1500 $cm^{-1}$, indicating a possible incomplete separation/extraction between the components in solution. This is expected with preliminary solvent extraction, and can be mitigated by chromatographic techniques such as gas chromatography or multiple combined washings of varying polarities

### MELANIN

Melanin extraction methods using 2 M NaOH yielded a dark brown supernatant shown in Figure 5. UV-Vis analysis (Figure 6) of the liquid showed peaks and maximum at 223.31 nm, and 244.88 nm, absorption values consistent with those present in melanin (8). Known absorption spectra of melanin produced by *Bacillus* bacteria show peaks at approximately 220 and 260 nm preceding a gentle slope towards larger wavelengths. The gentle slope shown in Figure 6, is a key characteristic for melanin, as absorbance present over the entire visible region is what provides the dark brown/black pigmentation. Due to the varying nature of its structure and the different types, UV-Vis spectra for melanin come in a variety of forms. The spectrum therefore allows for some limited distinguishability between these types.

The IR spectra obtained for the melanin extract (Figure 7) is consistent with , with absorption at labeled values described in the literature (19). The broad absorbance at 3300-3500 $cm^{-1}$ corresponds to -OH stretching. Other characteristic bands include the 1627 $cm^{-1}$ which is attributed to C=C, COO, and C=O, 1540 $cm^{-1}$ bands which are aromatic C=C, and 1051 $cm^{-1}$, which is C-O in phenols or carboxylic groups (19). The 1540 $cm^{-1}$ absorbance has been stated to correspond to peptide like structures (20) as well as stretches from quinoid structures present in allomelanin (21).

## PROTEOGENOMIC ANALYSIS

### CAROTENOID

The genome of *Planococcus* sp. CAU13 was analyzed using the BioCyc SmartTables function. Seven genes predicted to code for carotenoid biosynthesis pathway enzymes were found, including four distinct phytoene desaturase (*crtI*), two squalene synthase family enzymes, and a carotenoid biosynthesis enzyme. Furthermore, Moyo *et al.* compared the genetic sequences of 33 *Planococcus* strains known to produce carotenoids (2). Six genes (*crtM, crtN, crtNc, crtNb, gtf2,* and *agpat*) make up the 30-carotenoid biosynthetic gene cluster (BCG). In this study all six genes are present in all but two strains, with CAU13 being one of them that only showed *crtM, crtNc,* and *crtN* in its gene cluster.

### MELANIN

As *P. maritimus* is a tentative melanin producing bacterium with the 1,8-DHN pathway for allomelanin production, this specific pathway for melanin production was investigated (6). *P. maritimus* was determined to experimentally produce melanin through the 1,8-DHN pathway, however the published genome was not available at the time of this publication.

The enzyme described previously as PKSIII is the first step in this pathway. Using BioCyc's Whole Genome Blast tool, a preliminary BLAST was computed against the entire genome of CAU13 to determine if a homologous PKSIII enzyme is present. The PKSIII sequence used in the analysis was from *Sorangium cellulosum* produced from the soceCHS1 gene (7). The CAU13 locus_tag KW93_RS14965 translated to protein_id WP_018395371.1 produced significant alignment with the *S. cellulosum* chalcone synthase PKSIII with an E value of 0.0 and 90.17 percent identity through a BLAST search in the NCBI database.

The software predicts the function of the protein WP_018395371.1 as a chalcone synthase, a similar PKSIII

typically involved in producing the precursor to flavonoids (22). BLAST analysis using the PKSIII sequence from CAU13 revealed significant sequence similarty to the following enzymes: T4HN producing PKSIII enzymes from four other bacterial species, AUX21439.1 chalcone synthase *Sorangium cellulosum*, WP_033543849.1 type III polyketide synthase *Planococcus* sp. CAU13, WP_152152970.1 type III polyketide synthase *Bacillus aerolatus*, WP_394120825.1 type III polyketide synthase *Planococcus donghaensis*, WP_088007427.1 type III polyketide synthase *Indiicoccus explosivorum*. The protein sequences were then aligned using T-COFFEE as shown in Figure 8. (15)

The protein sequence alignment shown in Figure 8 shows a score of 95% similarity between the sequences, supporting the previous alignment identification as a T4HN producing PKSIII. The active site and 3D structure of the *Streptomyces coelicolor* A3 PKSIII have previously been determined (10). This active site is described to operate via Cys-His-Asn catalytic triad. All enzymes aligned show this consensus sequence at the positions described in the literature, C138, H270, and N303. To confirm that the active site residues are positioned at the correct orientation in space, AlphaFold software was used to predict the 3D structure of CAU13 PKSIII (Figure 9). This predicted model consists of the same general structure as the single monomer form of S. coelicolor A3.These three factors, the primary sequence similarity, the conserved active site, and the predicted 3D structure indicate that KW93_RS14965 codes for a T4HN producing PKSIII enzyme. Therefore CAU13 likely produces T4HN the first compound in the biosynthetic pathway for allomelanin.

## CONCLUSION

The spectra of the yellow carotenoid-based extract and the presence of a predicted phytoene desaturase within the potential C-30 BGC suggest that CAU13 produces xanthophyll type carotenoids consistent with other members of the genus. The dark pigmentation of CAU13 is likely due to the presence of allomelanin, a type of melanin produced in the 1,8-DHN pathway. Genetic and molecular analysis supports that a T4HN producing PKSIII is likely present due to a conserved active site and high protein homology to other characterized enzymes. UV-Vis and IR characterization of the dark extract support the determination of melanin, though full characterization of the pigment compounds will require analysis of a pure extract with the use of chromatographic techniques, GS-MS, or NMR spectroscopy. Carotenoids and melanin both provide potential fitness advantages by protecting against certain environmental factors, characterization of these compounds can help determine the specific role they play in CAU13 and better understand the organism's place within its ecosystem. Furthermore, biological pigments offer a wide array of industrial, medical, and research applications which should be explored.

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# The Effect of Laboratory Water Type on Bacterial Growth in Prepared Media

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## ABSTRACT

For life on earth, water serves as a reactant in metabolic pathways and as the universal solvent. In its role as a solvent, the use of distilled or even ultrapure water is often specified when preparing growth media. In preparation instructions, the rationale for this choice is rarely given. We wondered if water could be too pure, lacking essential trace elements not provided by the medium powders. Conversely, could tap water still retain compounds that would inhibit microbial growth? We hypothesized that, with current water purification methods, tap water would be suitable for preparing media in the microbiology laboratory. To test this hypothesis, we prepared two commonly used media, Nutrient Broth and Tryptic Soy Broth, in water from three sources: tap, deionized, and ultrapure. We then measured growth of three bacteria commonly used in the microbiology teaching laboratory. Growth was observed in all media preparations. There was no inhibition of growth when tap water was used to prepare the media. For *Escherichia coli* grown in Nutrient Broth, use of tap water appeared to lead to increased growth. Growth in media prepared with ultrapure water did not significantly differ from that in media prepared with tap water, with the one exception, or deionized water. The use of high purity water will remain important when the exact composition of a medium is required. However, our results suggest that tap water is adequate when preparing media for general purposes such as establishing working cultures for short-term use.

**Keywords**: growth medium preparation, bacterial growth, ASTM water type, water quality, microbial growth

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# INTRODUCTION

Microbial cultures in a laboratory setting are supported by a growth medium – a liquid or solid material that contains all the necessary nutrients for growth to occur (25). Ideally, these nutrients are provided in both the type and form in which they are biologically available to the microorganism of interest. Determining the correct form and type of nutrient is not always simple. In the early days of microbiology, bacteria were grown in liquid culture using meat infusions, such as would be used to prepare bouillon, or other extracts of biological material. Pasteur describes attempts to culture the causative agent of anthrax in meat broth, beer yeast extract, and even urine (11).

These complex and rich media typically supported good growth, but they impeded efforts to understand the details of microbial metabolism because their specific composition was unknown. Pasteur formulated a semi-defined growth medium composed of sugar, ammonium salts, and yeast ash (18, 24). Shifts to even more chemically defined media then led to identification of specific nutritional requirements. For example, the opportunistic fungal pathogen *Candida albicans* requires biotin (2, 3). *Haemophilus influenzae*, a bacterial cause of otitis media and meningitis, requires two factors named V and X (19, 23), later identified as nicotinamide adenine dinucleotide (NAD) and heme, respectively (7, 12).

Beyond the nutrients needed for metabolism, an important component of all growth media is water. Water can be considered a nutrient itself in that it can be a reactant in metabolic processes such as hydrolysis of polysaccharides into their monosaccharide subunits. However, it could be argued that water is most important as a solvent. One aspect of biological availability mentioned above is solubility and water is the solvent for living things on Earth. Thus, all cells require water. Vegetative cells of bacteria, fungi, and protozoa are susceptible to dehydration and die unless they have some way to retain water.

ASTM International (formerly the American Society for Testing and Materials) classifies reagent water into four types, with each type designation based on allowable limits of factors such as total organic carbon, bacterial counts, sodium and chloride concentration, and pH (1). Type I is the most highly purified. Dissolved salts are present at the lowest concentrations of any water type so that conductivity is very low (0.056 μS/cm) and resistivity is very high (18 MΩ·cm). Although tap water does not fall into any of the designated reagent water types, it is tested for constituents that might affect human health.

Just as water quality affects human health, so it may affect microbial growth. Water that is too pure may lack essential trace elements. However, water that is not pure enough may contain impurities that, while within safe levels for human health, may nevertheless be toxic to microbes. For example, water may retain residual antimicrobial compounds used during water treatment (9). With respect to the presence of toxins, many of the studies highlighting the importance of water purity in culture medium were done in the mid-20$^{th}$ century and before passage of the US Safe Drinking Water Act in 1974. Since then, water treatment approaches have improved (21) leading to the possibility that current water supplies are sufficiently pure to lack inhibitory agents.

The objective of this study was to determine if the quality of water used to rehydrate dry growth medium powders would affect bacterial growth. We hypothesized that tap water and deionized water would provide good growth, but that Type I water might result in decreased growth due to the lack of essential trace elements. We further hypothesized that, given current, improved, water treatment techniques, tap water would be sufficiently pure for use in typical laboratory cultures.

## METHODS

To test our predictions, we prepared two types of complex media, Nutrient Broth and Tryptic Soy Broth using water from three different sources: tap, house deionized, and ultrapure (Tap, DI, and MQ, respectively). We inoculated each growth medium with working stock cultures of one of three bacterial species commonly used in microbiology teaching laboratories (*E. coli, B. subtilis,* or *S. aureus*). We determined bacterial growth, as measured by turbidity of the culture, after 24 hours of incubation. Growth of each species in each medium was compared using ANOVA.

### BACTERIA ISOLATES AND CULTURE CONDITIONS

Growth media were commercially obtained (Neogen, Lansing, MI) as premixed powders. Nutrient Broth (NB, Acumedia NCM0110A) contains beef extract (3 g/L) and an enzymatic digest of gelatin (5 g/L). Powders from two different lots (103093B and 106127A) were used for comparison. Tryptic Soy Broth (TSB, Acumedia NCM0004A, lot 109771B) contains an enzymatic digest of casein (17 g/L), an enzymatic digest of soybean meal (3 g/L), sodium chloride (5 g/L), dipotassium phosphate (2.5 g/L), and glucose (2.5 g/L). Tryptic Soy Agar (Tryptic Soy Broth with 15 g/L agar) was used to culture working stocks of each isolate. Sodium chlo-

ride (S671-500) was obtained from Thermo Fisher Scientific (Fair Lawn, NJ).

Three water sources were used to prepare the growth media: Milli-Q (MQ), house deionized (DI), and tap (T). Milli-Q (Milli-Q$^{50}$ system, MilliporeSigma, Burlington, MA) provides ultrapure, or Type 1 water (1). The house deionized water system (Suez NA, Paramus, NJ) provides Type 2 water (Mike Ancy, SUEZ Water Technologies & Solutions, personal communication).

Growth media were prepared according to the manufacturer's specifications. Experimental broth media were prepared by dissolving growth medium powder in one of the three water sources, then transferred as 4 mL aliquots to glass culture tubes (13 × 100 mm, Thermo Fisher), capped, and sterilized by autoclaving (15 minutes, 121 °C, 15 psi, Priorclave, London, UK). TSB tubes for working stock cultures were prepared with DI water. Tryptic Soy Agar (TSA) plates were prepared by adding the powder to house DI water, heating to dissolve the agar, then autoclaving. Following sterilization, the medium was tempered to 50 °C in a water bath and then aseptically transferred to plastic Petri dishes (15 × 100 mm, MIDSCI, Valley Park, MO) and left to solidify.

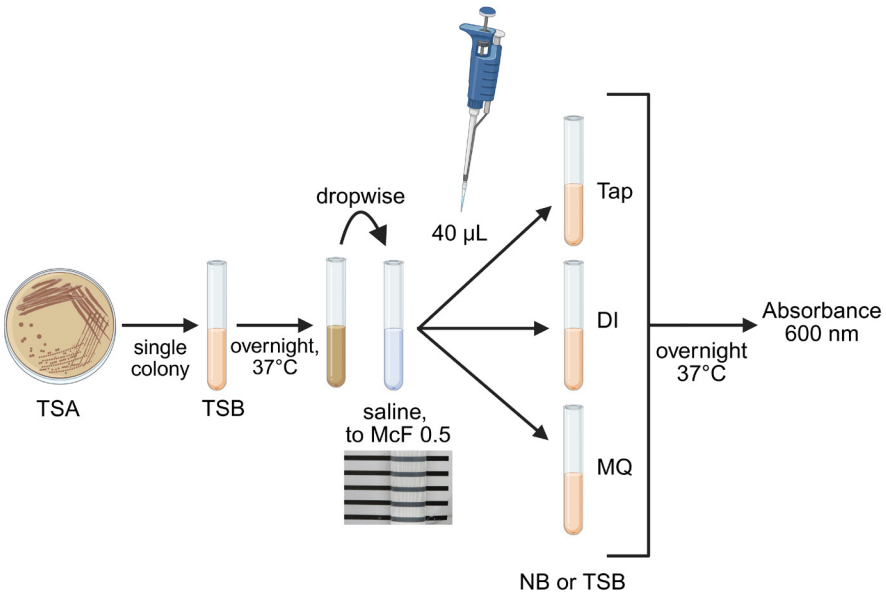
Three bacterial species commonly used in the microbiology teaching laboratory were used in this study: *Escherichia coli* (ATCC 35218), *Bacillus subtilis* (ATCC 6051), and *Staphylococcus aureus* (ATCC 29213) All were obtained from the American Type Culture Collection (Manassas, VA). *E. coli* is a Gram-negative rod. *B. subtilis* and *S. aureus* are both Gram-positive, with *B. subtilis* having a rod morphology and *S. aureus* being a coccus. We reasoned that use of multiple species would decrease the possibility of species-specific results being observed. Bacterial isolates are maintained as frozen stocks (CryoCare™, Key Scientific, Stamford, TX) at –80 °C. Prior to use in experiments, each isolate was grown from frozen stocks on TSA plates.

## EXPERIMENTAL DESIGN

For each organism, an isolated colony from the Tryptic Soy Agar stock plate was used to inoculate a TSB working stock tube. These working stock cultures were incubated overnight at 37 °C. The overnight culture suspension was transferred dropwise to 4 mL of sterile saline (0.85% w/v NaCl in DI water) until the turbidity matched a 0.5 McFarland Turbidity Standard (14). From this 0.5 McFarland saline suspension, 40 µL was used to inoculate each experimental TSB tube (Figure 1). All cultures were prepared in triplicate.

## FIGURE 1.

*Experimental design.*




Overnight cultures in Tryptic Soy Broth (TSB) were prepared for each bacterial isolate by inoculating with an isolated colony from the Tryptic Soy Agar (TSA) working stock plate and incubating at 37 °C. The culture suspension was transferred dropwise to 4 mL of sterile saline until the turbidity matched a 0.5 McFarland Turbidity Standard (inset). Each growth medium, prepared in each water type, was inoculated with 40 µL of the saline suspension. Figure created with BioRender.com.

**TABLE 1.**

*Turbidity measurements (600 nm) for bacterial cultures grown in Nutrient Broth (NB) or Tryptic Soy Broth (TSB) prepared in tap (T), deionized (DI), or MilliQ™ (MQ) water. Data are presented as the arithmetic mean ± sample standard deviation.*

| | *E. coli* | | | *B. subtilis* | | | *S. aureus* | | |
|---|---|---|---|---|---|---|---|---|---|
| | T | DI | MQ | T | DI | MQ | T | DI | MQ |
| NB 1 | 0.262 ± 0.025 | 0.230 ± 0.017 | 0.240 ± 0.024 | 0.201 ± 0.032 | 0.111 ± 0.032 | 0.121 ± 0.054 | 0.488 ± 0.053 | 0.439 ± 0.018 | 0.432 ± 0.016 |
| NB 2 | 0.284 ± 0.025 | 0.199 ± 0.016 | 0.207 ± 0.036 | 0.141 ± 0.066 | 0.102 ± 0.019 | 0.114 ± 0.024 | 0.422 ± 0.071 | 0.389 ± 0.073 | 0.390 ± 0.058 |
| NB-C[a] | 0.273 ± 0.025 | 0.215 ± 0.022 | 0.223 ± 0.033 | 0.171 ± 0.057 | 0.106 ± 0.024 | 0.117 ± 0.038 | 0.455 ± 0.066 | 0.414 ± 0.055 | 0.411 ± 0.044 |
| TSB | 1.039 ± 0.047 | 1.030 ± 0.041 | 1.032 ± 0.055 | 0.412 ± 0.034 | 0.374 ± 0.057 | 0.387 ± 0.049 | 1.152 ± 0.037 | 1.230 ± 0.051 | 1.184 ± 0.025 |

[a]NB-C: combined NB trials

Normalizing the saline suspension to a turbidity standard ensured that each experimental culture tube for a given isolate received an equivalent inoculum. Cultures were incubated at 37 °C. After 24 hours of incubation, turbidity was measured spectrophotometrically (Spectronic 20D+, Model 333183, Thermo Electron Corporation). Absorbance at 600 nm was measured and recorded, using a tube of uninoculated medium as a reagent blank.

For each days' measurements, the mean and standard error of the mean were calculated. Each trial with each organism grown in each medium prepared with water from each of the three different sources, was done in triplicate. Data sets were compared by two-way ANOVA using SPSS® (10). Pairwise comparisons were performed using Bonferroni post-hoc tests. For all comparisons, a = 0.05. Box plots were generated using BoxPlotR (20).

## RESULTS

On first inspection, each organism appeared to grow equally well regardless of which type of water was used to prepare the medium (Table 1). Closer inspection of the results revealed three additional trends. First, in the NB trials, it appeared that turbidity was slightly higher in the Tap water tests than in the DI and MQ water tests. This trend was less apparent in the TSB trials. Second, all absorbances in TSB were considerably higher than those in NB. Depending on the water source and organism, this increase was 2.5 – 4.8-fold. Finally, there appeared to be differences among the organisms. *B. subtilis* tended to exhibit less growth in both media and in all three water types when compared to the other two organisms. *S. aureus* exhibited the greatest amount of growth in comparison to both other bacteria in each medium and for each water type.

To determine if the observed growth reflected a specific medium formulation (i.e., was lot-specific), growth in two separate lots of Nutrient Broth was measured and compared. Individual cases involving each organism in each medium in each water type were analyzed. (e.g., *E. coli* NB-1 and NB-2 in tap water; *E. coli* NB-1 and NB-2 in deionized water; etc.) An F-test and a two-tailed t-test were conducted for each pair to compare the variance and means (a = 0.05), respectively. Results of these tests indicated that variances were the same ($p > 0.05$ for all cases) and means were the same ($p > 0.05$ for all cases). Since the variances were equivalent, data from the six NB trials were combined (Table 1, NB-C).

Two-way ANOVA analysis of the data sets supported this conclusion. For each organism, a two-way ANOVA was performed using the three water sources and the three media (two NB lots and the TSB trials). This type of analysis allows us to compare all cases without the increased probability of Type I error (rejecting the null hypothesis that means are equivalent when it is actually true) that would result from performing multiple *t*-tests. Results of this analysis showed that variances were equal for all groups, and that the means of the two NB lots were not different ($p = 1.0$, 0.703, 0.102 for *E. coli*, *B. subtilis* and *S. aureus*, respectively).

The two-way ANOVA analysis was repeated using the combined NB data in the medium comparison. The tests of between-subjects effects resulted in a low *p*-value and a high partial eta squared ($\eta^2_p$) value for each organism (Table 2). This suggests a large effect due to the medium (NB vs TSB). When comparing water sources, the results were less clear. For all organisms, no significance was associated with water source ($p > 0.05$). However, for effect size estimates, suggested benchmarks are given as 0.01 (small effect), 0.06 (medium effect), 0.14 (large effect) (5). Our estimate calculations all exceed the 0.01 benchmark, suggesting some effect due to water.

Based on these results, we wondered if the suggested water effect was influenced by including the medium comparisons. Thus, we performed one-way ANOVAs comparing

## TABLE 2.

Between-subject effects test from a two-way ANOVA comparing growth in two different media (NB and TSB) prepared in water from three sources (tap, DI, MQ) (a = 0.05).

| Organism | Medium | | Water | |
|---|---|---|---|---|
| | Significance (*p*) | Effect Estimate ($\eta^2_p$)[a] | Significance (*p*) | Effect Estimate ($\eta^2_p$) |
| *E. coli* | > 0.001 | 0.993 | 0.131 | 0.176 |
| *B. subtilis* | > 0.001 | 0.910 | 0.068 | 0.226 |
| *S. aureus* | > 0.001 | 0.984 | 0.620 | 0.044 |

[a]Effect size rule of thumb: 0.01 (small), 0.06 (medium), 0.14 (large)

only the water groups – that is, a separate ANOVA for each medium. Post-hoc comparisons of the one-way ANOVAs showed that when the bacteria were grown in TSB, water had no effect (no difference in mean turbidity, Table 3). This was also true when *B. subtilis* and *S. aureus* were grown in NB (Table 4). When *E. coli* was grown in NB, mean turbidity for Tap water was significantly different from that in DI (p = 0.006) and MQ (p = 0.02). These results are graphically presented as box plots (Figure 2).

To determine if there was an actual growth difference between the organisms, we performed a 2-way ANOVA for each medium, looking at the effect of water and organism. For TSB, there was an effect associated with the organism ($p > 0.001$, $\eta^2_p = 0.989$). This analysis indicated no effect due to water ($p = 0.163$, $\eta^2_p = 0.018$), which is consistent with the ANOVAs described above. For NB, there was also an effect associated with the organisms ($p > 0.001$, $\eta^2_p = 0.905$). The effect of water noted above was seen again in this analysis ($p = 0.001$, $\eta^2_p = 0.279$).

## DISCUSSION

We originally hypothesized that water type (tap, deionized (DI), or ultrapure (MQ)) would affect growth of bacteria in two media typically used in microbiology teaching laboratories. Specifically, media prepared with tap and DI could provide good growth, while media prepared with ultrapure water might show less growth due to missing trace elements not supplied by the medium. We also considered, while formulating our hypothesis, that growth in tap water would be lower due to some residual presence of a growth inhibitor. Taken together, our results suggest that the water types tested did not significantly affect bacterial growth.

**Water type did not significantly affect growth of the test bacteria.** Several previous studies identified negative effects of water type on bacterial growth. Butterfield (4) suspended bacteria in distilled water or water containing sodium bicarbonate (0.5 g/L) for 15 or 30 minutes. They reported a decrease in growth relative to control when bacteria were subsequently inoculated onto standard growth medium. They observed that growth returned to baseline when the suspension water was supplemented with potassium phosphate or with a combination of potassium phosphate, calcium chloride, magnesium sulphate, and ferric chloride. The authors surmised that the cells were damaged by the hypotonic environment, similar to the rupturing of protozoa in the same conditions. Price & Gare (16) reported the presence of an inhibitor of bacteria growth in distilled water. In this case, the water tested was used to rinse tubes prior to the addition of growth medium. The authors concluded that the rinse water left the inhibiting compound on the glass. This inhibitor was not identified but was evidently removed by degassing the water or by passing the water through a mixed bed ion-exchange column. Hoadley & Cheng (9) studied the survival of

## TABLE 3.

*Post-hoc tests of significance (Bonferroni) comparing Tryptic Soy Broth medium prepared in the three water sources (a = 0.05).*

| | **Significance (*p*)** | | |
|---|---|---|---|
| **Comparison** | *E. coli* | *B. subtilis* | *S. aureus* |
| tap → DI | 1.000 | 1.000 | 0.144 |
| tap → MQ | 1.000 | 1.000 | 1.000 |
| DI → MQ | 1.000 | 1.000 | 0.593 |

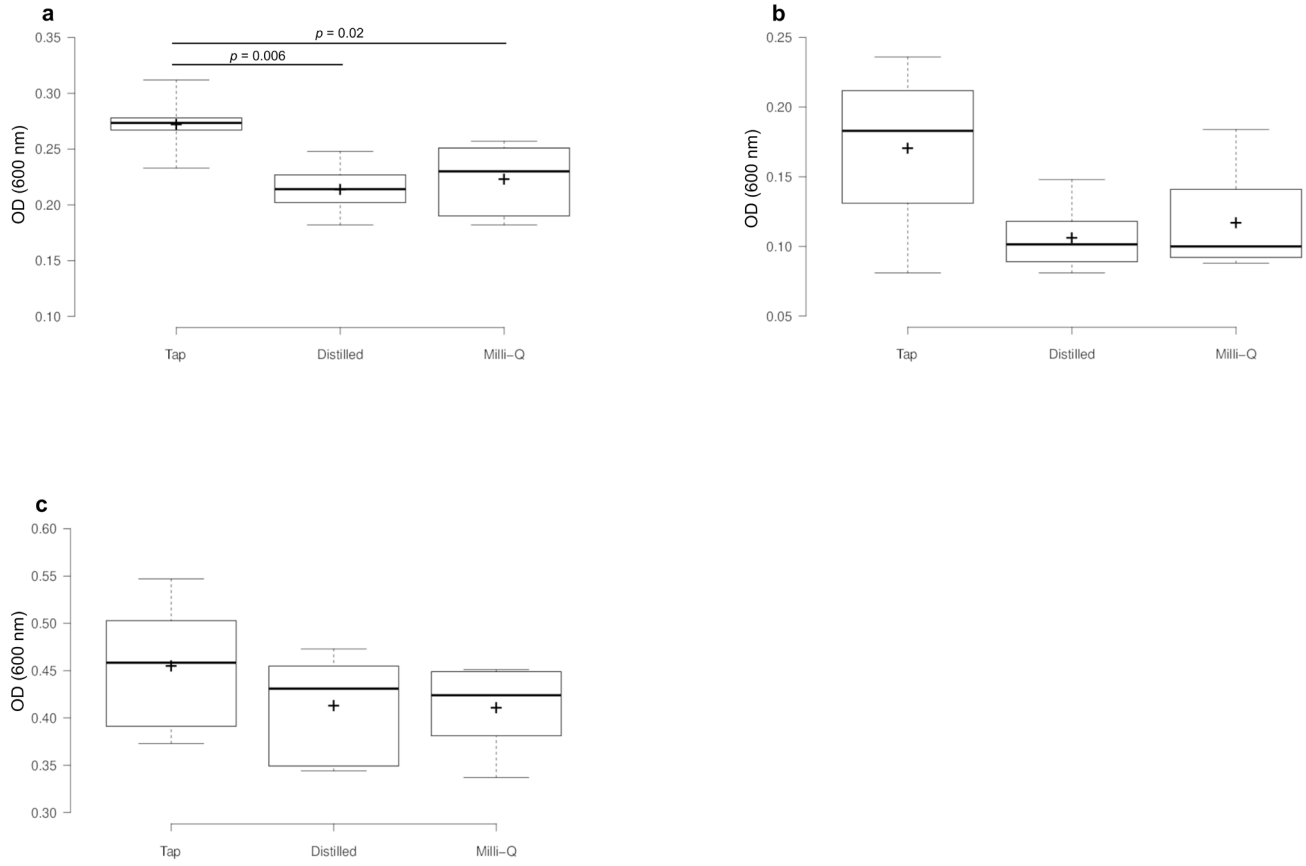
## TABLE 4.

*Post-hoc tests of significance (Bonferroni) comparing Nutrient Broth medium prepared in the three water sources (a = 0.05).*

| | **Significance (*p*)** | | |
|---|---|---|---|
| **Comparison** | *E. coli* | *B. subtilis* | *S. aureus* |
| tap → DI | 0.006 | 0.051 | 0.661 |
| tap → MQ | 0.020 | 0.125 | 0.575 |
| DI → MQ | 1.000 | 1.000 | 1.000 |

## FIGURE 2.

*Comparison of growth in Nutrient Broth prepared with tap, deionized (DI) or Milli-QTM (MQ) water. (a)* Escherichia coli, *(b)* Bacillus subtilis, *(c)* Staphylococcus aureus.




Center lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend to minimum and maximum values; crosses represent sample means. $n = 6$.

bacteria in a water environment and observed a rapid loss of viable bacteria when suspended in tap water. They were unable to recover viable bacteria after 4 – 8 hours. However, in all of these reports, researchers used water as a sample diluent and not to prepare the growth medium, distinguishing them from this study.

Our results provide no evidence that tap water contains anything toxic to the test bacteria. The statistical analyses suggested that differences in growth, as measured by turbidity, were an effect of the medium or the organism, but not of the water type used to prepare the medium. The exception to this was for *E. coli* grown in Nutrient Broth, in which growth in NB prepared with tap water differed from that in NB prepared with deionized water or ultrapure water (Table 1, Table 4, Figure 2). Visualization of the data by boxplot suggests this difference relates to the spread of data points for the *E. coli* trials (Figure 2, see Factors for considerations section below).

**TSB supported higher growth than NB did.** We observed that, for the bacteria tested, growth was higher in TSB than in NB. As water had no significant effect on growth, the difference between growth in NB and TSB likely stems from differences in composition. NB and TSB are both complex, meaning that the exact chemical composition is not known due to the inclusion of extracts from plants, animals, or yeasts. NB contains beef extract and enzymatic digest of gelatin. TSB contains enzymatic digest of casein (a primary protein derived from milk), enzymatic digest of Soybean meal, NaCl, $K_2HPO_4$, and glucose.

The addition of $K_2HPO_4$ to TSB may provide some buffering capacity for the medium. Static cultures used in this

study leads to fermentative metabolism, which could lower culture pH below optimum levels. However, amino acids and other extract components can also provide some buffering. Thus, systematic measurement of culture pH before inoculation and after incubation would be needed to determine a growth-culture pH correlation. A more likely explanation is the inclusion of glucose (0.25%) in TSB. Because it can enter glycolysis at the first step, glucose as a catabolic substrate may serve as a preferred substrate for these species and provide an initial head start to microbial growth. Further experiments, adding glucose to NB to determine effects on growth, would test this hypothesis.

***Bacillus subtilis* grew less than *E. coli* or *S. aureus* regardless of water type or medium.** A final observation from the data was that *B. subtilis* had lower amounts of growth than *E. coli* or *S. aureus* regardless of medium or water type (Table 1). One possible explanation for this is lack of a critical nutrient. Although they did not specifically test *B. subtilis*, Proom & Knight (17) reported a requirement for thiamine for various *Bacillus* species, when using ammonium as a nitrogen source and amino acids as carbon sources. However, since both media used in this study were complex, composition analysis would be needed to determine if either was thiamine deficient or if thiamine supplementation would improve *B. subtilis* growth. Another explanation is the growth temperature used in this study. According to ATCC, the recommended growth temperature for *B. subtilis* is 30 °C, while that for both *E. coli* and *S. aureus* is 37 °C. Thus, it is possible being above optimal temperature was sufficient to inhibit growth. Replicating the *B. subtilis* trials when grown at 30 °C would test this possibility.

**Factors for consideration.** Our results raised several factors for consideration regarding our study. These include the sample sizes, testing conditions, the types of media and organisms used in the study.

**Sample size and testing conditions.** Each trial was performed in triplicate over different days and working cultures. This was done to normalize results over potential changes in laboratory conditions and was sufficient to conduct statistical analysis of the results. However, additional replicates could help clarify suggested differences, such as *E. coli* growth in NB prepared in tap water compared to DI and MQ (Table 1, 4). Further, we tested only one strain for each species. Extension of the experiments to involve other strains are needed to demonstrate that results are not strain-specific. Additionally, we performed all experiments using a single preparation of each growth medium. Although we would not expect to see differences in batches of media prepared using the same stock bottle of powder, running these experiments would confirm that expectation. Finally, we did not measure water parameters such as conductivity, pH, chlorine content, dissolved solids, etc. While these values are reported by the city water treatment facility, values may differ between the testing facility and the tap. Conductivity is measured by the Milli-Q[50] system, but no other parameters are reported by that system. These water parameters were included in our discussions during experimental design, and we reasoned that any differences in the water itself would be eliminated after the addition of the medium. Systematically tracking these parameters both before and after addition of medium in future studies would confirm this reasoning and identify possible variation over time.

**Use of Complex Media.** As noted above, both NB and TSB are complex media, with sources of growth factors and trace elements needed by bacteria and other microorganisms. We chose these media because they are commonly used to prepare stock cultures for microbiology teaching and research labs. Defined media, in which the exact chemical composition is known, may be more sensitive to levels of varying levels of solutes, particularly trace metals, due to the water source.

**Use of Non-fastidious Organisms.** The study organisms were chosen because they are standard cultures grown in a microbiology teaching lab. All are non-fastidious organisms, meaning they do not require special nutritional additives or growth conditions. Fastidious organisms, which have more specialized nutritional requirements, might be more sensitive to the presence or absence of solutes, such as trace metals, depending on the water source.

## CONCLUSIONS

To our knowledge, this is the first study looking at the effect of water type (quality) on laboratory growth of bacteria since a study conducted by Geldreich & Clark (8) in 1965, in which they reported on a method for testing the suitability of water produced by various distillation methods. To demonstrate the efficacy of a suitability test they had developed, they compared nine different distilled water samples, using the same minimal growth medium to determine if the respective water samples were toxic to growth of *Aerobacter aerogenes*.

Most papers in the literature specify distilled water for media preparation. In 1966, Ellner et al. (6) specified the use of distilled water for media formulation without further elaboration. In 1974, Neidhardt et al. (15) specified glass-distilled water for preparation of a defined medium, again providing no rationale. The Difco Manual (25) specifies rehydration in "purified water" or water without any "added substance (pg. 5, 22)." Historically, the rationale for using distilled water to

prepare growth medium is not explicitly stated, though it is reasonable to suppose the concern is the presence of environmental toxins remaining in unpurified tap water. A note from Oxoid Technical Support (22) advises against the use of tap water for hydration of culture media given the presence of impurities including calcium, magnesium, and metal ions in addition to chlorine and fluorine, the latter two of which can "alter characteristics of selective media." They ended the note with a focus on the extremely "variable" ion content present in tap water. However, it is unclear as to when these recommendations were first developed or if they were ever retested. Even in the literature, the advice to use distilled water is not entirely consistent. For example, McClung (13) describes preparation of Nutrient Broth by dissolving powers (peptone & beef extract) in tap water. Furthermore, the paper goes on to note that "distilled water is often listed for this medium [NB] but is unnecessary."

We do not mean to suggest that water type is unimportant. Neidhardt et al. (15) noted that variation in growth media and conditions hindered the full understanding of *E. coli* and *S. typhimurium* physiology. Given their observations, the conventional wisdom alluded to above of using the highest purity water that is economically feasible (e.g., deionized water for rehydrating growth media) makes sense. Moreover, everyone experiences some changes in tap water quality in times of drought, flooding, or water main damage. Nor are downstream purification systems immune from water quality fluctuations. Recently, our institution experienced degradation of water from our in-house deionization system because of contamination during building renovations.

Nevertheless, our results suggest that the current-day quality of tap water generally is sufficient for day-to-day lab stock cultures and can be used if the DI system is unavailable. This can be particularly beneficial for resource-limited labs in which purchase and maintenance of water purification systems is cost-prohibitive. Even when such systems are in place, they may fail or otherwise have some interruption of water production. In these types of circumstances, our results demonstrate that useful cultures can still be grown, allowing for students to experience and master basic microbiological concepts and skills.

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# Metabolites from *Aspergillus sclerotiorum* fungal growth medium are cytotoxic to and inhibit cell cycle progression in *Drosophila melanogaster* S2 cells in culture

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**Keywords**: *Aspergillus sclerotiorum*, *Drosophila melanogaster*, entomopathogenic, cytotoxic, cell cycle, ochratoxin A, apoptosis

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## ABSTRACT

*Aspergillus sclerotiorum* has been identified as an entomopathogenic fungus with potential as a biological control agent, yet its cellular effects on insect hosts are not well characterized. We hypothesized that filtered growth medium from *Aspergillus sclerotiorum* cultures contains one or more fungal metabolites that damage cells in culture. To explore possible mechanisms, we tested cell-free fungal culture filtrates on *Drosophila melanogaster* S2 embryonic cells. Filtrates from older cultures were strongly cytotoxic and showed a reduced percentage of live S2 cells comparable with positive controls for cell death. Similar effects were seen in mouse fibroblast L929 cells. At lower concentrations, the fungal medium did not cause overt cytotoxicity but did consistently reduce total S2 cell numbers. Few dead cells were present in these conditions, indicating the medium impaired replication rather than inducing cell death. This reduction was accompanied by a decreased expression of two cell cycle regulators. Proliferating cell nuclear antigen (PCNA) fell to less than 6% of controls, while Cyclin A dropped to below 30% of controls at the most dilute treatment. These results indicate that *A. sclerotiorum* secretes one or more metabolites that decrease cell viability at higher concentrations and inhibit proliferation at lower concentrations, potentially by interfering with cell cycle progression. ELISA analysis of ochratoxin A production in this strain showed only minimal levels of the toxin, suggesting other unidentified fungal factors may be the source of these observations. These findings advance understanding of fungal–insect interactions and suggest that soluble compounds produced by *A. sclerotiorum* may contribute to its pathogenicity in insect hosts.

## INTRODUCTION

Entomopathogenic fungi (EPF) have the potential to be used as biopesticides due to their direct cuticle penetration of insect hosts. EPFs also play an important role in natural ecosystems as plant growth promoters, rhizosphere colonizers, plant disease antagonists, and endophytes (16). Fungi naturally occurring in termite habitats are promising biological control agents, due to their adaptation in such environments (30). The most effective and thoroughly studied EPF, *Metarhizium anisopliae*, has been suggested as a potential termite control agent (15, 22). However, biocontrol agents can be limiting, in that they work more slowly than chemical pesticides (16).

Previous work has shown *Aspergillus sclerotiorum* to be pathogenic to both *Incisitermes minor* and *Reticulitermes hesperus* termites (10). These two species of western termites are among the 5 pest species found in the United States (25), making *A. sclerotiorum* a potential biocontrol agent for termites. In addition, as a biosafety level 1 (BSL-1) agent, *A. sclerotiorum* is unlikely to be significantly harmful to most humans, although it may pose some threat to immunocompromised people (1, 2, 11, 18, 24).

The mechanisms of termite infection and pathogenicity by *A. sclerotiorum*, as well as the fungal host range, remain largely unknown, although a pilot study showed that *Drosophila melanogaster* insects were susceptible to *A. sclerotiorum* infection (unpublished data). In an effort to study insect host-fungal interactions on the cellular level, *D. melanogaster* S2 embryonic cell line (23) were used in many of these studies, since these cells are well-studied, easy to maintain, and a termite cell line does not yet exist. Additionally, a mouse cell line (L929) was used to test whether the fungus had similar effects on mammalian cells.

Here we present findings of the effects of treating S2 cells with dilutions of filtered, cell-free *Aspergillus sclerotiorum* growth medium. S2 cells showed increased cytotoxicity from older cultures of filtered fungal growth medium at higher concentrations. The same cytotoxic phenomenon was observed when mouse L929 cells were treated with the fungal growth medium, which led to our hypothesis that the fungus secretes a molecule(s) that is cytotoxic. When S2 insect cells were treated with more diluted fungal growth culture, however, insect cells did not show cytotoxicity, but they had a marked decrease in cell numbers per sample compared to control treated cells. It was then hypothesized that the fungus secretes a molecule(s) that arrests S2 cells in cell cycle. An analysis of Proliferating Nuclear Cell Antigen (PCNA) and Cyclin A protein levels from treated S2 cells confirmed that they had lower levels of these proteins, indicating that cells were arrested in cell cycle. Further studies indicated that Ochratoxin A (OTA), a mycotoxin made by many *Aspergillus* fungi (27), was not made at high levels by the strain of *A. sclerotiorum* used in this study. This study furthers the understanding of the insect host and pathogen interactions between *D. melanogaster* insect cells and *A. sclerotiorum* fungus.

## MATERIALS AND METHODS

### CELL LINES AND FUNGAL CULTURES.

The *Drosophila melanogaster* cell line, S2 (23), was obtained from the *Drosophila* Genomics Resource Center (Bloomington, Indiana) and the American Type Culture Collection (ATCC, Rockville, Maryland). S2 cells were grown and maintained at room temperature (approximately 20-25 ℃) in S2 medium containing Gibco Schneider's Drosophila Medium, 10% heat-inactivated fetal bovine serum (FBS), and with or without 1× penicillin-streptomycin (medium from ThermoFisher Scientific, Waltham, Massachusetts; FBS from Sigma-Aldrich, St. Louis; pen-strep from HyClone Laboratories, Logan, Utah). The mouse fibroblast cell line, L929, was also obtained from ATCC (Rockville, MD). L929 cells were grown at 37° C and 5% $CO_2$ and maintained in L929 medium containing Dulbecco's Modified Eagle's Medium, 10% heat-inactivated FBS, 10 mM HEPES buffer, and 1× penicillin-streptomycin (Medium from Caisson Labs, Smithfield, UT; HEPES from Sigma-Aldrich).

Two strains of *Aspergillus sclerotiorum,* APU strain (10) and Huber strain (16892, ATCC, Rockville, MD)(12), and *Aspergillus alliaceus* (NRRL-315, Agricultural Research Service (ARS) Culture Collection, Peoria, Illinois) were all grown and maintained on Sabouraud dextrose agar (SDA) plates (BD Diagnostics, Franklin Lakes, New Jersey) or in Sabouraud dextrose broth (SDB) (Sigma-Aldrich, St. Louis). *A. sclerotiorum* APU strain was used in all experiments, and only indicated where Huber strain was also used. Glycerol stocks from APU and Huber strains of *A. sclerotiorum* were made and stored at -80℃.

### FUNGAL GROWTH MEDIUM AND DILUTIONS.

*A. sclerotiorum* APU or Huber strain was inoculated from a glycerol stock stored at -80℃ into approximately 30 mLs of Sabouraud dextrose broth (SDB) in 50 mL conical tubes with loosened lids. Tubes were then grown in a shaker incubator at 25℃ at 220 rotations per minute (rpm) in the dark, along with 30 mLs of uninoculated control medium. Inoculated or matched uninoculated tubes were incubated for either 4, 7, or 10 days. After this, fungal or control tubes were centrifuged at 3,000 rpm for 15 minutes, followed by removal of supernatant, which was then filter-sterilized using a syringe with a 0.22 µm filter. Dilutions of fungal growth medium and uninoculated control medium were made in S2 or L929 medium and immediately used in S2 or L929 cell experiments, except for one experiment analyzing Cyclin A or PCNA proteins where fungal growth medium and uninoculated control medium as described above were lyophilized. In this case, fungal cultures or uninoculated controls were incubated for 10 days, centrifuged, and the supernatants were filter-sterilized, frozen, and then lyophilized overnight. The resulting powders were kept at -20°C until reconstituted to the original 1X in S2 medium, filter-sterilized, and these were further diluted into 1:2.5, 1:5, and 1:10 working concentrations in S2 medium.

### CELL VIABILITY AND CELL QUANTIFICATION ANALYSIS.

S2 and L929 cells were each seeded in 24-well plates at 2 x $10^5$ cells per well 1-2 days before treatment with fungal growth medium. For loosely adherent S2 cells, half the medium was taken off and replaced with the appropriate treatment. For adherent S2 or L929 cells, all medium was taken off and replaced with the appropriate treatment. S2 cells were treated with either 0.8 µM actinomycin D (ActD) or 33.3 µg/mL cycloheximide (Sigma-Aldrich, St. Louis, MO) as positive controls for cytotoxicity. L929 cells were treated with ActD as a positive control for cytotoxicity. Cells were incubated for 48 hours with these various treatments and then analyzed. For each experiment, 3-4 wells were used per treatment.

Cell viability and quantification were measured using propidium iodide staining followed by flow cytometry. After 48 hours, S2 cells or L929 cells from each well were collected into a separate microfuge tube (L929 cells were removed with trypsin first), then centrifuged at 4,000 rpm for 2 minutes. The supernatant was removed and the cell pellets were washed twice with phosphate buffered saline (PBS) before being resuspended in 100 µl of FACS solution (3% heat-inactivated FBS and 0.09% (w/v) sodium azide in PBS, pH 7.4-7.6, then filter sterilized through a 0.22 µm pore membrane). Then 5 µl of propidium iodide (PI) (Sigma-Aldrich, St. Louis) was added to each sample and vortexed before being read on the BD Accuri C6 Plus flow cytometer, using the BD Accuri C6 software (BD Biosciences, San Jose, California). The number of cells in each sample was quantified by the total number of events counted in two minutes at a slow rate (14 µl/minute) by flow cytometer. At least 10,000 events were recorded per sample, as previously suggested (7). To measure cell viability, a gate was set to exclude cell debris based on side scatter versus forward scatter plots, then a second plot of PI fluorescence vs forward scatter was generated, which showed two distinct cell populations (modified slightly from (7)). The population with lower fluorescence was determined to be the live cells, while the population with the higher fluorescence was determined to be the dead cells. Live cells were expressed as a percent of the total cells in the well. Samples for each treatment were

averaged. Depending on the experiment, either one-way or two-way ANOVA with replication was performed, followed by Tukey's HSD post-hoc test ($\alpha = 0.05$), or Student's t-tests were performed to determine one-tailed *p* values.

### *Cyclin A and PCNA Western blots*

*Drosophila melanogaster* S2 cells were passaged regularly and maintained in Schneider's Drosophila Medium described above, but without antibiotics. For experimental and control treatments, S2 cells were seeded in 6-well plates at a density of $1.6 \times 10^6$ cells per well in a total of 2 mLs, and allowed to adhere for 24 hours before treatment.

Cells were harvested, spun down, washed in cold phosphate-buffered saline with 0.1% Tween-20 (PBS-T), and re-suspended in 250 µl of RIPA lysis buffer containing 1X broad spectrum protease inhibitor cocktail and 1mM EDTA (Boster Bio, Pleasanton, CA). Lysates were kept on ice and periodically vortexed for 30 minutes before being stored at -20°C. Protein concentrations were determined using the Qubit Broad Range Protein Assay (Thermo Fisher Scientific, Waltham, MA). 10 µg of each sample was denatured in SDS sample buffer at 95°C for 5 minutes before being loaded onto 12% tris-glycine SDS-PAGE gels with 6 µl Precision Plus Protein Kaleidoscope ladder (both from Bio-Rad, Hercules, CA) and separated by electrophoresis. Proteins were then transferred to nitrocellulose membranes using the Bio-Rad Turbo Blot system for 7 minutes. Membranes were blocked overnight in 1% bovine serum albumin (BSA) in 0.1% PBS-T.

Primary monoclonal anti-Cyclin A (A12), anti-α-Tubulin (12G10), and anti-PCNA (PC10) were diluted 1:1000 in 1% BSA in 0.1% PBS-T and incubated either for 1 hour at room temperature or overnight at 4°C. Blots were probed with HRP-conjugated anti-mouse secondary antibody diluted 1:1000 in blocking buffer. Signals were visualized using Amersham ECL detection reagents (GE Healthcare, Chicago, IL, USA) on a Bio-Rad ChemiDoc imaging system. Membranes were stripped and re-blocked before reprobing for α-Tubulin. A12 and 12G10 antibodies were purchased from Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA; PC10 antibody from NeoBiotechnologies, Irvine, CA; secondary antibody from Sigma-Aldrich, St. Louis, MO.

All blots were analyzed using Bio-Rad Image Lab software. Bands were background-subtracted and normalized to corresponding α-Tubulin signals. Cyclin A and PCNA blots were performed in duplicate from cells treated with fresh liquid fungal growth or control media, and once with cells treated with reconstituted lyophilized media before analysis. One representative PCNA blot is shown.

## QUANTIFICATION AND STATISTICAL ANALYSIS OF WESTERN BLOTS

The primary metric for the analysis of PCNA and Cyclin A was the band intensity from the protein of interest that had been normalized to the housekeeping protein, α-Tubulin, to determine a relative expression. The resulting relative band intensity for each fungal growth medium was compared to its respective uninoculated control. For each dilution in the Cyclin A blots, the null hypothesis of no change was tested with a one-sample, two-tailed Student's t-test against $\mu = 1.0$ (df = $n$–1), with $\alpha = 0.05$. n = 3 biological replicates. No outliers were excluded.

### *Ochratoxin A detection in* Aspergillus sclerotiorum

Coconut cream agar plates were prepared by boiling 300 mL of water with 50 g of coconut cream powder (Kara, Pulau Sambu, Singapore) and 12 g of biological grade agar (Bioreagents, Hummelstown, PA) followed by sterilization in the autoclave. Fungal cultures of *A. alliaceus*, *A. sclerotiorum* APU strain, and *A. sclerotiorum* Huber strain from glycerol stocks were inoculated onto the plates and incubated at 24 °C in the dark for at least 4 days. Plates were inverted and observed under UV light (365 nm) for OTA's blue fluorescence (13). The Huber strain of *A. sclerotiorum* was reported to produce no detectable OTA by this method, while the NRRL-315 strain of *A. alliaceus* was shown to produce high levels of OTA by this method (12), and thus were chosen as control strains.

Ochratoxin A competitive ELISA kit (Antibodies.com, St. Louis, Missouri) was used to quantify the ochratoxin A from 10-day cultures of *Aspergillus alliaceus, A. sclerotiorum* APU, and *A. sclerotiorum* Huber, grown in Sabouraud dextrose broth (Sigma-Aldrich, St. Louis). Fungal samples were filter-sterilized using a syringe with a 0.22 µm filter and used for the ochratoxin A kit according to manufacturer's instructions. Two wells on the ELISA plate were used for each sample and then averaged; three wells of each standard were used and averaged to generate the standard curve. The $R^2$ value of the standard curve was 0.9689.

## RESULTS

### S2 CELL VIABILITY WAS REDUCED AFTER TREATMENT WITH 10-DAY FUNGAL GROWTH MEDIUM COMPARED TO 4-DAY FUNGAL GROWTH MEDIUM

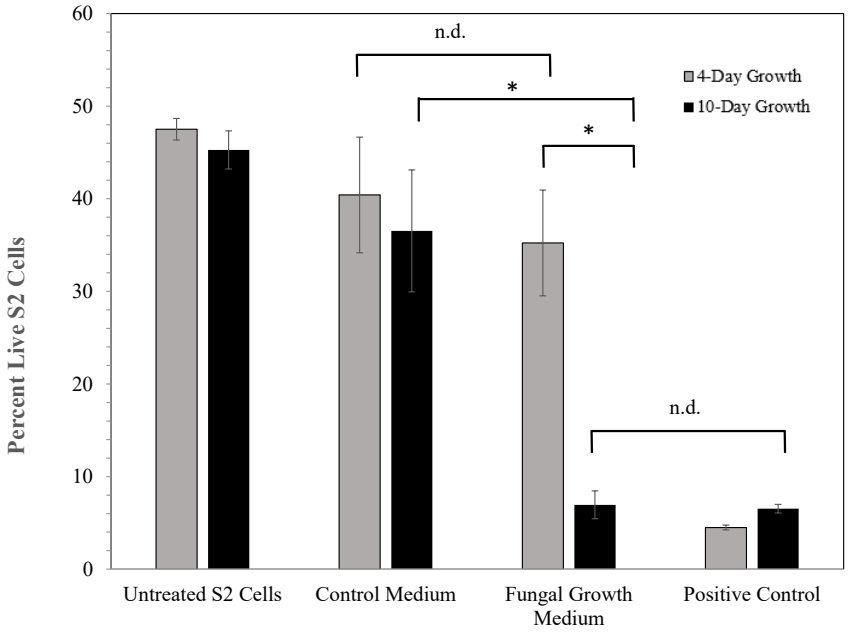
The effect of cell-free, filtered fungal growth medium from 4-day or 10-day cultures on *Drosophila* S2 cell viability was investigated and shown in Figure 1. With 4-day fungal growth medium treatment, there was no statistically significant difference between the percentage of live S2 cells treated with uninoculated control medium compared to cells treated with *A. sclerotiorum* fungal growth medium after 48 hours (40.4% vs 35.2%, respectively, Fig. 1). By contrast, S2 cells treated with 10-day fungal growth medium treatment resulted in significantly fewer live S2 cells than uninoculated control-treated S2 cells (7.0% live S2 cells vs 36.5% live S2 cells, Fig. 1). The 10-day fungal growth treatment had a similar effect on S2 viability as actinomycin D, the positive control for cytotoxicity (7.0% vs 6.5%, not statistically different, Fig. 1). The percentage of live S2 cells treated with 4-day and 10-day fungal culture media were also significantly different (35.2% vs 7.0%, respectively), showing a decrease in S2 cell viability as the fungal cultures aged.

### FUNGAL GROWTH MEDIUM IS CYTOTOXIC TO A MOUSE CELL LINE *IN VITRO*

The cytotoxicity of fungal growth medium was also tested on a mammalian fibroblast cell line, mouse L929 cells. Figure 2 shows that 48 hours after L929 cells were treated with 10-day *A. sclerotiorum* growth medium, only 15.8% of cells were viable, whereas 79.0% of the L929 cells treated with uninoculated medium remained viable. This was a significant reduction in viability in the mouse cells (Tukey's post-hoc test, $p<0.05$). The viability of L929 cells treated with fungal growth medium was not statistically different from cells treated with the positive control for cytotoxicity, actinomycin D.

## FIGURE 1.

*Treatment with 10-day* A. sclerotiorum *growth medium culture resulted in less S2 cell viability than 4-day* A. sclerotiorum *growth medium culture treatment.*




S2 cells were either untreated, treated with diluted (1:2.5) uninoculated Sabouraud dextrose broth (Control Medium), diluted (1:2.5) *A. sclerotiorum* 4-day or 10-day filtered growth medium (Fungal Growth Medium), or a positive control for 48 hours before staining with propidium iodide and analyzed by flow cytometry. Positive controls were 33.3 μg/mL cycloheximide in the 4-day fungal growth trial, and 0.8 μM actinomycin D in the 10-day fungal growth trial. Each bar represents the mean of three samples ± SEM. * indicates $p < 0.05$ (two-way ANOVA with replication ($F(3,16) = 6.09$, $p = 0.0058$), followed by Tukey's HSD post-hoc test); n.d. indicates not statistically significant ($p > 0.05$).

## DILUTED FUNGAL GROWTH MEDIUM LOST ITS CYTOTOXICITY TO S2 CELLS, BUT RESULTED IN A DECREASE IN TOTAL S2 CELL NUMBERS

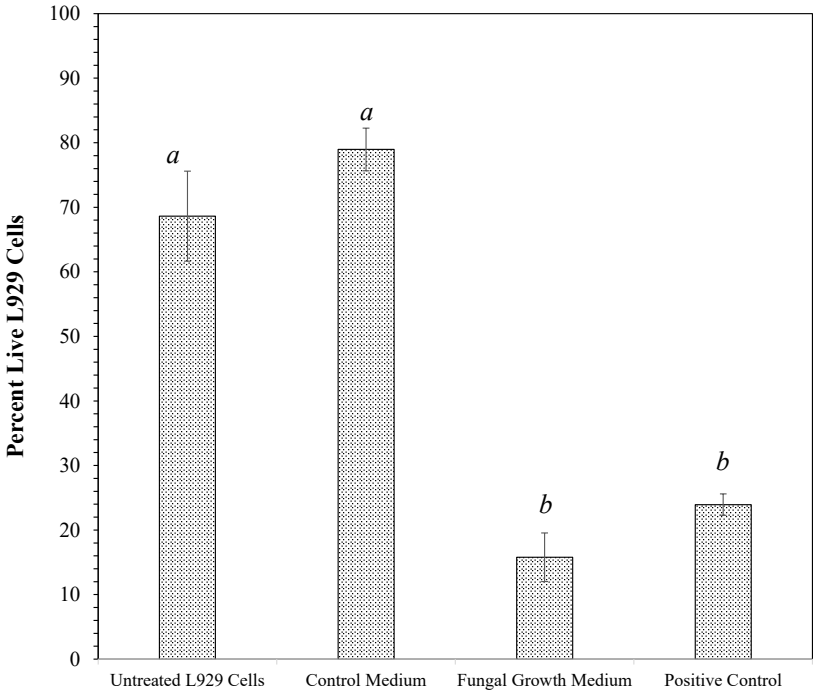
Figure 3 shows the effect that diluted filtered *A. sclerotiorum* 7-day fungal growth medium had on S2 cell viability as well as total S2 cell numbers after 48 hours. The 1:5 dilution resulted in a significantly lower average percent of live cells compared to the 1:5 dilution of uninoculated control medium (35.7% vs 77.9%, $p < 0.001$) (Fig. 3A). The 1:10 dilution also resulted in a significantly lower average percent of live cells compared to its uninoculated control, although less so (53.4% vs 77.5%, $p = 0.03$) (Fig. 3A). The average total number of cells treated with the 1:5 dilution of fungal growth medium was also drastically lower compared to the same dilution of uninoculated control medium (approximately 24,000 cells vs 175,000 cells, $p < 0.001$, Fig. 3B). When the same fungal growth medium was diluted 1:20, there was no longer a significant difference between the percentage of live cells compared to the uninoculated control medium (Fig. 3A). However, the numbers of cells present in each well treated with 1:10 and 1:20 dilutions of fungal growth medium were still significantly lower than the numbers of cells in each well treated with diluted uninoculated control medium ($p < 0.001$) (Fig. 3B).

## DECREASE IN PCNA PROTEIN LEVELS FOLLOWING DILUTED FUNGAL GROWTH MEDIUM

S2 cells were treated with uninoculated control medium or inoculated fungal growth medium at three dilutions (1:2.5, 1:5, and 1:10), and the PCNA protein expression was analyzed (Figure 4). An analysis of relative band intensities in Figure 4 showed that PCNA expression in cells treated with fungal growth medium was approximately 22% of the control at the 1:2.5 dilution, approximately 27% at the 1:5 dilution,

## FIGURE 2.

A. sclerotiorum *growth medium was cytotoxic to mouse L929 cells.*




Mouse L929 cells were either untreated, treated with diluted (1:2.5) uninoculated Sabouraud dextrose broth (**Control Medium**), diluted (1:2.5) *A. sclerotiorum* 10-day filtered growth medium (**Fungal Growth Medium**), or treated with a positive control (0.8 μM actinomycin D) for 48 hours before staining with propidium iodide and analyzed by flow cytometry. Each bar represents the mean of four samples ± SEM. One-way ANOVA was performed ($F$ (3,12) = 52.29, p = 3.66E-07). Groups marked with different letters (*a* vs *b*) differ significantly using Tukey's HSD post-hoc test ($p < 0.05$).

and approximately 6% at the 1:10 dilution levels compared to their respective diluted uninoculated control medium.

### DECREASE IN CYCLIN A PROTEIN LEVELS FOLLOWING EXPOSURE TO DILUTED FUNGAL GROWTH MEDIUM

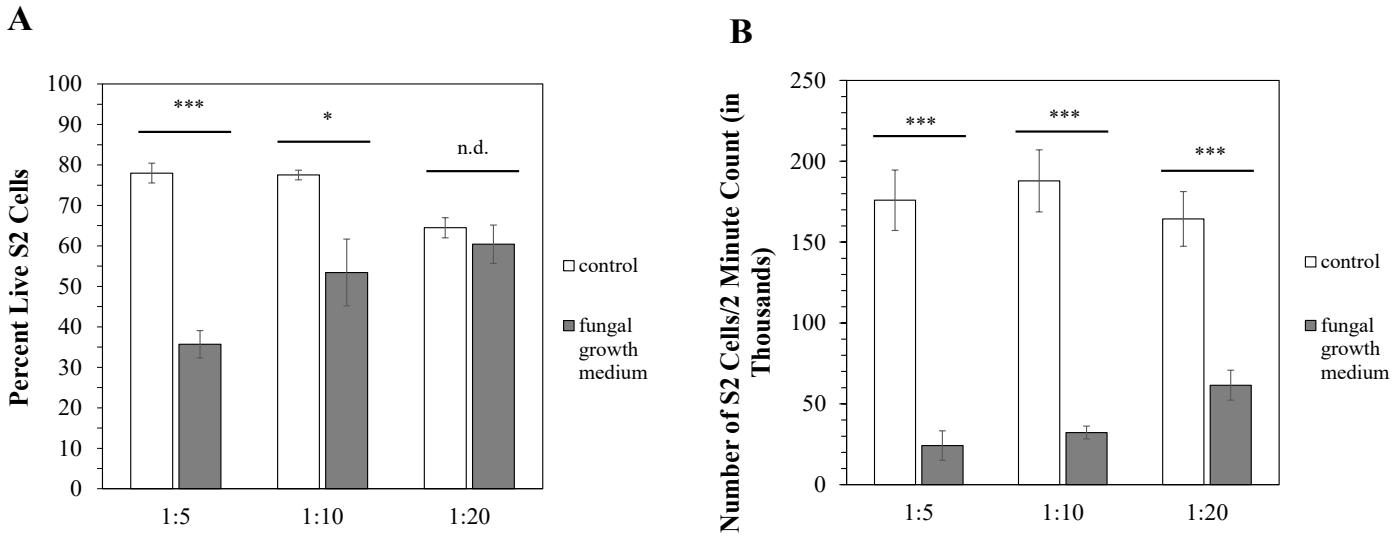
S2 cells showed a consistent decrease in Cyclin A expression following exposure to fungal growth medium at both 1:5 and 1:10 dilutions. Cyclin A levels were found to be approximately 65% at 1:5 dilution, and approximately 29% at 1:10 dilution compared to their respective diluted uninoculated medium controls (Table 1). While the reduction at the 1:5 dilution was not statistically significant ($p$=0.127), the reduction in Cyclin A levels at the 1:10 dilution was found to be statistically significant ($p$=0.037).

### *ASPERGILLUS SCLEROTIORUM* PRODUCED ONLY VERY LOW LEVELS OF OCHRATOXIN A (OTA)

To determine whether ochratoxin A (OTA) is produced by the APU strain of *A. sclerotiorum*, the fungus was first grown on coconut cream agar (Figure 5A). This medium induces the production of OTA, which fluoresces blue under ultraviolet (UV) light. On coconut cream agar plates, *Aspergillus alliaceus* displayed strong blue fluorescence consistent with OTA production (13), whereas both the Huber and APU *A. sclerotiorum* strains lacked visible OTA-associated blue fluorescence (Figure 5A). Quantification by competitive ELISA assay confirmed these observations: *A. alliaceus* produced a high OTA concentration of 14.8 ng/mL when grown for 10 days in fungal medium, while the *A. sclerotiorum* strains APU and Huber produced low levels of OTA, 1.3 ng/mL and 1.4 ng/mL, respectively (Figure 5B). These levels were only slightly above the background of the uninoculated control broth (0.3 ng/mL).

## FIGURE 3.

*More concentrated fungal growth medium was toxic to S2 cells, while more diluted fungal growth medium had no effect on S2 cell viability but resulted in decreased total numbers of S2 cells.*




S2 cells in culture were treated for 48 hours with varying dilutions of fungal growth medium as shown from a 7-day culture, and then stained with propidium iodide and analyzed by flow cytometry. The controls were diluted, uninoculated Sabouraud dextrose broth medium diluted in S2 medium. Each bar represents the mean of four samples ± standard error. *** indicates $p < 0.001$ (one-tailed, Student's t-test); * indicates $p < 0.05$; **n.d**. indicates not statistically significant ($p > 0.05$). This experiment was conducted 3 separate times, with one representative experiment shown. A. Live and dead cells were determined by propidium iodide staining, and expressed as a percent of live cells within each sample. B. Total cell numbers in samples were counted from 2-minute runs.

## TABLE 1.

*Decrease in Cyclin A Levels in S2 Cells Treated with Fungal Growth Medium Vs Control Medium.*

| Dilution of Medium | Relative Cyclin A Band Intensity [a] | % Change | *P* value [b] |
|---|---|---|---|
| 1:5 | 0.653 ± 0.238 | - 34.7% | 0.127 |
| 1:10 | 0.287 ± 0.245 | -71.3% | 0.037 |

[a]Band intensities were normalized to α-tubulin within each lane, then expressed as fungal growth medium/ control medium ratios for each dilution, with mean shown ± standard deviation.
[b] Two-tailed t-test comparing normalized band intensities, fungal growth medium vs control medium. $\mu = 1.0$ (df = n–1), with $\alpha = 0.05$. n = 3 biological replicates.

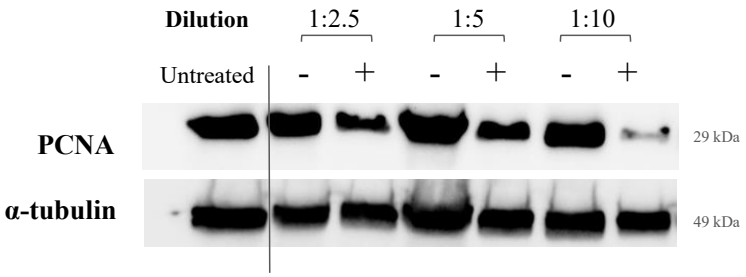
## DISCUSSION

We have previously shown that a novel fungus, *Aspergillus sclerotiorum,* is entomopathogenic to two species of western termites (10), although the mechanisms of infection and pathogenesis are still largely unknown. In this report, filtered cell-free *A. sclerotiorum* growth medium from either older cultures or at more concentrated dilutions were shown to be toxic to cells from the S2 *Drosophila* insect cell line (Figs. 1 and 3A). In addition, treatment with fungal growth medium was also shown to decrease L929 mouse cell viability (Fig. 2). These together strongly suggest that *A. sclerotiorum* secretes one or more cytotoxic compounds or metabolites, and that the concentration of toxic molecule(s) accumulate over time in culture medium. Although currently unknown, it would be interesting to determine whether the fungal growth medium retains its pathogenicity in live insects, as well.

The toxicity seen in cells treated with *A. sclerotiorum* fungal medium is likely caused by one or more mycotoxin or other fungal metabolite(s). Some strains, but not others, of *A. sclerotiorum* tested have previously been shown to produce the mycotoxins ochratoxin A (OTA) and ochratoxin B (OTB) (6, 12, 13, 26), which are toxic to both insects (8) and mammalian cells and humans (reviewed in 28). It is unlikely, however, that the cytotoxicity seen in fungal growth medium-treated S2 cells (Fig. 1 and 3A) or L929 cells (Fig. 2) reported in this study are due to OTA, since only very low levels of OTA were detected in fungal growth medium by competitive ELISA (Fig. 5B). While the S2 cell line was treated with filtered fungal growth medium in these studies, we have previously worked with live termites inoculated directly with viable fungal spores (10). It remains unclear whether low levels of OTA in *A. sclerotiorum* APU strain contribute to pathogenicity of the fungus in the whole insect host.

## FIGURE 4.

*PCNA expression in S2 cells decreases following* A. sclerotiorum *growth medium exposure.*



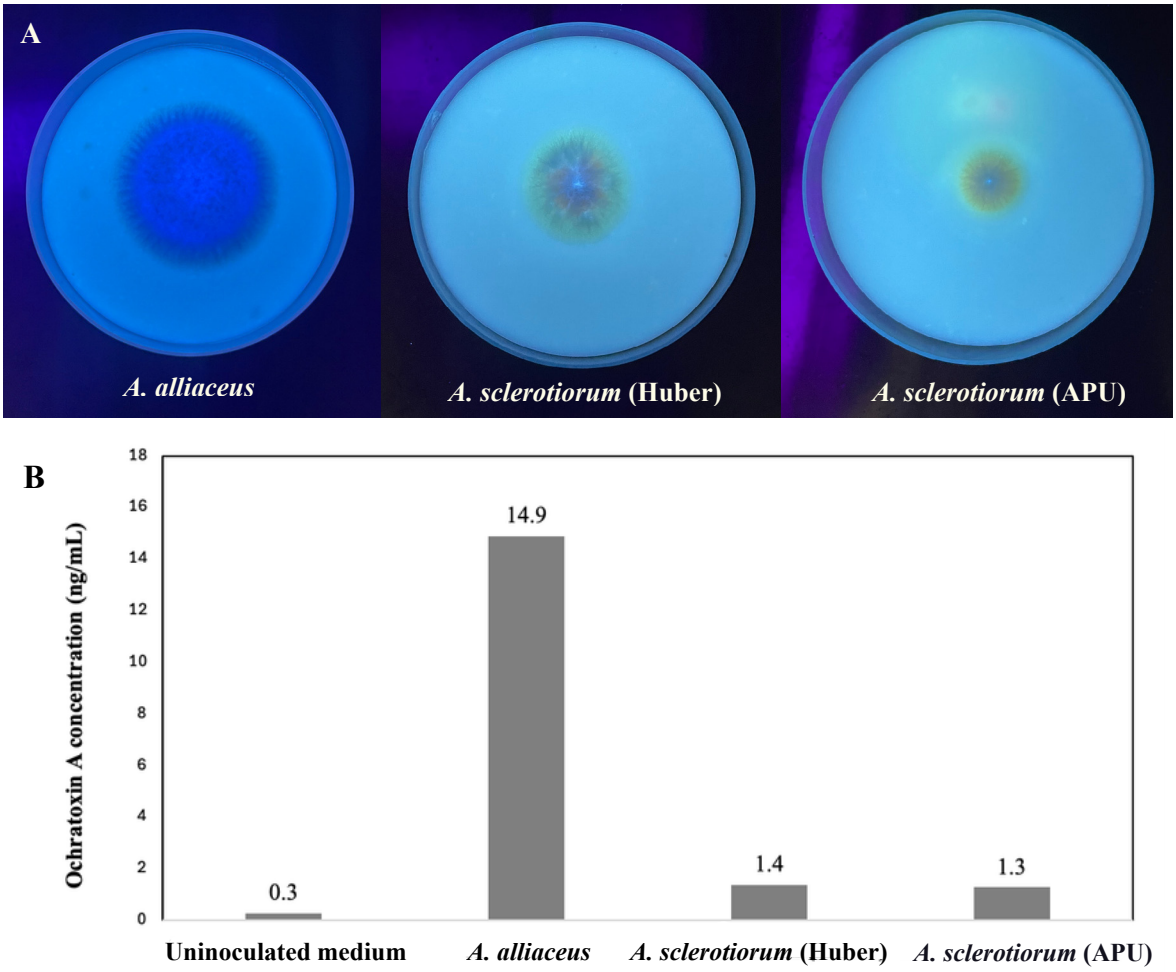

S2 cells were treated for 48 hours with either fungal growth medium (+) or control medium (-) at 1:2.5, 1:5, and 1:10 dilutions. Untreated cells remained in their regular S2 media for the 48 hours. Whole-cell lysates were immunoblotted for PCNA and band intensity was normalized to the housekeeping protein, α-Tubulin.

While there was no visible blue fluorescent OTA seen on the coconut cream agar plates in Figure 5A, there was a light green fluorescent molecule seen in both strains of *A. sclerotiorum*, which could indicate the presence of another mycotoxin normally found in *Penicillium,* citrinin (19), although whether it is citrinin remains to be determined. Other potential *A. sclerotiorum* metabolites that might play a role in the cell toxicity seen in these experiments could include aspochracin, shown to have anti-insect properties in silkworm larvae (20) and reported to be produced by *A. sclerotiorum* (4); or sclerotiamide, isolated from the sclerotia of *A. sclerotiorum* with anti-insect properties (8,29), although different forms of sclerotiamide have shown variable cytotoxicity when tested in human tumor cell lines (9,29). It is also possible that one or more molecules in *A. sclerotiorum* growth medium that have not yet been identified contributed to or was responsible for the decreased cell viability seen in our studies. More work needs to be done, however, to identify relevant specific fungal metabolites that mediate the effects reported here.

As *A. sclerotiorum* fungal growth medium was diluted, S2 cells showed less toxicity, where the percentage of live cells per sample treated with 1:20 dilutions was not signifi-

## FIGURE 5.

*Low levels of ochratoxin A (OTA) detected in* Aspergillus sclerotiorum *strains.*




(A) Growth of *A. alliaceus* (positive control, 4 days), *A. sclerotiorum* APU strain (6 days), and *A. sclerotiorum* Huber strain (6 days) on coconut cream agar. Plates were inverted to visualize under ultraviolet (UV) light (365 nm). OTA fluoresces blue-green under UV light. (B) Competitive ELISA showing OTA concentrations (ng/ml) in 10-day culture medium or uninoculated control medium. The mean of two ELISA wells from the same sample is shown for each bar.

cantly different from 1:20 diluted negative control medium ($p$ = 0.23, Fig. 3A). Interestingly, however, after 48 hours of treatment with diluted fungal growth medium, there were fewer S2 cells in every well, clearly visible by light microscopy (data not shown) and measured by a 2-minute sample run by flow cytometry (Fig. 3B). This data suggests that there was a molecule(s) secreted by *A. sclerotiorum* that inhibited S2 cell mitosis. Analysis of the expression of molecular markers specific to phases of the cell cycle, PCNA and Cyclin A, assisted in determining if the reduced cell numbers observed were due to fungus-mediated impaired proliferative capacity.

PCNA expression levels were decreased in S2 cells after treatment with fungal growth medium in all dilutions tested (1:2.5, 1:5, 1:10) compared to uninoculated medium controls, with the largest decrease in PCNA level unexpectedly seen in the most diluted samples (Fig. 4), where cells did not show high levels of cytotoxicity (Fig. 3A). Previous data did show the 1:2.5 and 1:5 doses to be cytotoxic rather than anti-proliferative (Fig. 3A), and the moderate decrease in PCNA expression at those higher doses could have been related to more severe cell injury and cellular stress. The pronounced and unexpected reduction in PCNA at the 1:10 dilution is likely more consistent with a coordinated decrease in proliferative activity than with generalized cytotoxicity. This may be the result of one or more fungal metabolites directly inhibiting cell cycle whose effect can be seen more dramatically when cells are not undergoing cytotoxicity; for instance, more cellular machinery could be intact in these cells that directly or indirectly causes PCNA degradation. More experimentation would be necessary to ascertain this, however. It is also possible that the decrease in PCNA reflects cellular stress rather than direct inhibition of cell cycle by one or more fungal metabolites. PCNA is a conserved DNA clamp protein that acts as a co-factor for DNA polymerases and is essential for the replication of genetic material in cells. It is also used widely as a marker of active DNA synthesis (reviewed in 5). In *Drosophila*, PCNA has been shown to be structurally and functionally similar to mammalian PCNA, with its expression limited only to cells that are actively dividing (21). Therefore, it can be considered an appropriate measure of S-phase activity, and more broadly, overall proliferative activity. This reduction in the expression of PCNA is consistent with the reduced percent viable cells seen in fungal growth medium-treated S2 cells (Fig. 3B).

The decrease in Cyclin A expression could possibly be attributed to a cell cycle phase redistribution within the cell population, resulting in fewer treated cells found in late S/G2-M, or it could indicate a checkpoint or other regulation that reduces Cyclin A abundance. Cyclins are regulatory proteins that activate kinases and are present at different levels during different phases of the cell cycle. This allows them to be used to identify specific points in cell cycle progression (14). Cyclin A binds with Cdk1 to drive the S/G2/early-M progression and is essential for mitotic entry (17). When cells experience replication stress or DNA damage, checkpoint systems are activated to delay the division of damaged DNA, either for repair or programmed cell death. In *Drosophila* S2 cells, this G2/M checkpoint is mainly controlled by the kinase Grapes (Grp), the fly equivalent of human Chk1 (reviewed in 3). However, the Cyclin A decrease observed in this study cannot be assigned to either direct inhibition of Cyclin A by the fungal growth medium versus secondary consequences from cell cycle control mechanisms.

Here we show for the first time that filtered fungal growth medium from *A. sclerotiorum* is cytotoxic to both insect and mouse cells, and that low levels inhibit insect S2 cells from proceeding through the cell cycle. While more work is needed to determine the specific molecule(s) secreted by *A. sclerotiorum* that mediate these effects, this report furthers the understanding of these host-pathogen interactions, and gives us an *in vitro* method for investigating them.

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# Metagenomic analysis of metalworking fluids from three different automotive manufacturing facilities

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## ABSTRACT

Metalworking fluids are used in metal machining and cutting as lubricants and coolants, and sometimes for their anti-corrosion properties. Metalworking fluids are commonly used in automotive manufacturing plants where potential contamination by bacteria poses public health concern. This study was conducted to evaluate the usefulness of metagenomics in determining the identity of bacteria and the diversity and richness of bacterial communities in metalworking fluids. Five metalworking fluid samples from three different plants, located in two different U.S. states, were obtained. A protocol was optimized to isolate DNA from metalworking fluids. A metagenomic analysis was performed by sequencing the V3-V4 regions of the 16S rRNA gene. Bacterial communities were analyzed and the diversity and richness of the samples were determined. Genera such as *Mycobacterium* and *Pseudomonas* were relatively abundant in samples from some plants, whereas other genera such as *Comamonas* and *Acinetobacter* were more abundant in other samples. All five samples were found to have bacterial communities that included opportunistic pathogens, emphasizing the importance of surveillance and treatment of metalworking fluids as important steps in addressing a potential public health concern for automotive manufacturing workers.

**Keywords**: metalworking fluids, metagenomics, *Mycobacterium*, *Pseudomonas*

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## INTRODUCTION

The microbiology of metalworking fluids used in industrial manufacturing remains poorly understood in the automotive industry, where metalworking fluids play many different roles in metalworking, machining, and metal-forming processes as coolants, lubricants, and for metal chip removal at the metal-tool interface (1, 2). There are many metalworking fluid formulations, and water-based semi-synthetic fluids are most widely used (1, 3). Water-based semi-synthetic fluids are mostly composed of water, which dissolves components such as refined petroleum, emulsifiers, chlorine, phosphorus, biocides, and fatty acid derivatives. These chemicals enhance the lubricity and high temperature/pressure endurance of metalworking fluids. The various components of metalworking fluids can provide nutrients for microbial communities to become established, grow, and spread in metalworking fluid systems (1).

An interest in microbial contamination of metalworking fluids began in 1993 after 10 cases of hypersensitivity pneumonitis originated in machinists exposed to aerosolized metalworking fluids at an industrial plant in the United States (4). In 1995, the sputum of infected workers was found to have acid-fast bacteria. After further analysis, it was suspected that infections were associated with a new species of mycobacteria, *Mycobacterium immunogenum* (4). Additional cases of hypersensitivity pneumonitis were identified at eight additional automotive plants in the 1990s (5). This discovery piqued interest in how metalworking fluids can become contaminated, which other microbial populations are supported by their composition, as well as how we can protect the health of workers by preventing exposure to pathogenic microorganisms. Individuals in close contact with metalworking fluids, such as those who are working on a machining production line or those in charge of maintaining or treating metalworking fluids, are the individuals most at risk for negative health consequences. This exposure can either be in the form of inhalation of aerosols of contaminated metalworking fluids or by direct skin contact (2, 6).

The presence and growth of microorganisms in metalworking fluids originate in different ways, including initial contamination routes as well as continued support of the microbial communities from ineffective routine cleaning procedures (3). For example, metalworking fluids are most commonly diluted with tap water which typically serves as the baseline introduction of microorganisms into industrial systems and reservoirs. Other routes include contamination from the industrial settings, workers, and residual biofilms that can continually reestablish microbial communities (3). Metalworking fluids are known to host a wide diversity of microorganisms including *Legionella*, enteric bacteria, pseudomonads, fungi, and *Mycobacterium* sp. (1). Exposure to pathogens in metalworking fluids has been found to increase the risk of developing hypersensitivity pneumonitis, cancer, dermatitis, asthma, respiratory illness, and reduced pulmonary function (1, 2, 6).

Metagenomics is the study of all genomes of microorganisms isolated from a bulk sample. It is particularly useful for studying communities of microorganisms in various environments, such as human stools or water samples (7). Development in next generation sequencing and bioinformatics tools have allowed researchers to use these techniques for the analysis of complex microbial communities (7). A few recent studies utilized metagenomic approaches to assess bacterial and fungal diversity of metalworking fluids from sites in Russia, China, and Switzerland (8, 9, 10). However, it does not appear that metagenomics has been utilized to analyze the microbial communities of metalworking fluids used specifically in automotive manufacturing facilities.

Currently, routine microbial testing of metalworking fluids mostly consists of cultivation methods, which require long incubation periods and may not correctly identify all opportunistic pathogenic bacteria (2). Consequently, the goal of this study was to evaluate whether DNA can be isolated from automotive metalworking fluids and then processed for further metagenomic analysis. Our objective was to conduct a metagenomics analysis on the isolated DNA using 16S rRNA DNA sequencing, and to assess the bacterial taxonomy of the metalworking fluids. The overall aim was to develop a robust approach that can be utilized by stakeholders to evaluate bacterial communities, including pathogens, in metalworking fluids.

## MATERIALS AND METHODS

### SAMPLING SITES

Samples (1 L each) of water-soluble semi synthetic metalworking fluid were collected by local employees from five different reservoirs in three automotive plants located in two midwestern U.S. states (Table 1). All metalworking fluids tested are used for machining automotive parts. The samples were collected in sterile polypropylene plastic bottles from the bulk fluid of the reservoirs in September and October 2025 and immediately shipped to the lab for analysis.

**TABLE 1.**

*Sample sites for metalworking fluid and their use.*

| Plant | Reservoir | Location | Used for: |
|---|---|---|---|
| 1 | 1A | Ohio | Machining automotive discs and drums |
| 1 | 1B | Ohio | Machining automotive transmission parts |
| 2 | 2A | Indiana | Machining automotive engine parts |
| 2 | 2B | Indiana | Machining automotive engine parts |
| 3 | 3 | Indiana | Machining automotive transmission parts |

## DNA ISOLATION

To concentrate suspended microorganisms, 200 mL of each metalworking fluid was thoroughly mixed and centrifuged (4,100 X g, VWR MegaStar 1.6). The concentrated samples were further centrifuged in microtubes (10,000 X g, Fisher Accuspin MicroR). Pellets ranging from 250-450 µg were obtained through this process from each reservoir. DNA isolation was performed on the concentrated metalworking fluid samples (pellets) using the FastDNA Spin Kit for Soil (MP Biomedicals) and the recommended protocol from the manufacturer with some modifications. In brief, this consisted of lysing the bacterial cells in the pellets using a lysing matrix solution and a homogenizer (BeadBug Microtube Homogenizer) for 80 seconds. This was followed by protein precipitation, DNA binding to a spin filter, washing steps, and DNA elution as per the manufacturer protocol. The concentration and quality of the DNA was measured at 260 nm and 280 nm using a Jenway 7315 spectrophotometer. Low DNA purity (low $A_{260}/A_{280}$) was a common issue with these samples. To increase DNA purity, the duration of protein precipitation step in the FastDNA kit was doubled.

## METAGENOMICS ANALYSIS

A DNA library of the V3-V4 regions of the 16S rRNA gene was constructed and sequenced by CD-Genomics (Shirley, NY) using a paired-end (PE) method on the Illumina NovaSeq X platform and published primers (11). The sequencing data were processed using the QIIME 2 platform (11). Determination of the bacterial composition of each sample was performed by read merging, Amplicon Sequence Variant

**TABLE 2.**

*Data statistics of the quality control of the DNA sequences from each metalworking fluid sample.*

| Sample | Raw PE[a] | Combined PE[b] | Qualified PE[c] | Nochime PE[d] | Base(nt) | AvgLen (nt)[e] | GC[f] | Q20[g] | Q30[g] |
|---|---|---|---|---|---|---|---|---|---|
| Reservoir 1A | 104072 | 103408 | 101888 | 100074 | 42548688 | 425.17 | 53.80% | 98.90% | 95.98% |
| Reservoir 2A | 112822 | 112196 | 110291 | 108226 | 44403784 | 410.29 | 56.95% | 98.92% | 95.96% |
| Reservoir 3 | 106161 | 105648 | 104095 | 103970 | 42517134 | 408.94 | 57.47% | 99.04% | 96.37% |
| Reservoir 1B | 104190 | 103115 | 100990 | 94647 | 40645857 | 429.45 | 51.05% | 98.58% | 94.88% |
| Reservoir 2B | 73931 | 73467 | 72181 | 61527 | 25395940 | 412.76 | 54.71% | 98.88% | 95.85% |

[a]Raw PE: Unprocessed Paired-End (PE) reads obtained from the sequencing platform.

[b]Combined PE: PE reads where both ends of a single DNA sequence are sequenced.

[c]Qualified PE: PE reads that have been reviewed by the prediction algorithm.

[d]Nochime: Clean Tags (no chimeras) following QC.

[e]AvgLen: the average length of the Clean Tags.

[f]GC: the percentage of guanine (G) and cytosine (C) base pairs.

[g]Q20 and Q30: the percentage of bases with a quality score equal to or higher than 20 (error rate <1%) and 30 (error rate <0.1%).

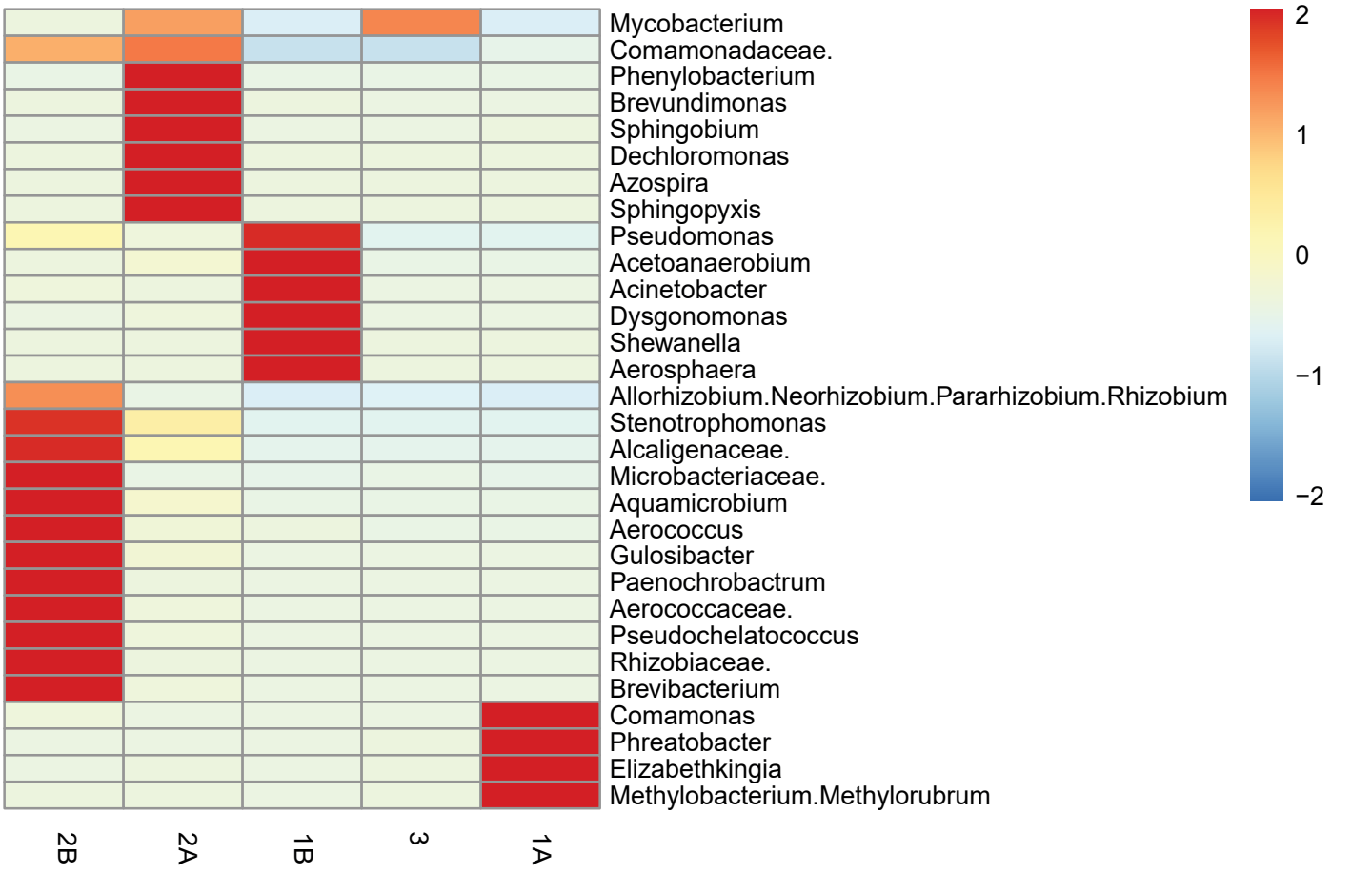
(ASV) detection, species annotation using a pre-trained Naïve Bayes classifier (trained on the Silva 138 99% ASVs), and abundance analysis (12). Four different indices were calculated to assess alpha diversity: Chao1 and Ace estimate the number of species in a community; Shannon and Simpson account for both richness and evenness of a community (13). Beta diversity analysis used three algorithms, binary Jaccard, Bray-Curtis dissimilarity, and unweighted UniFrac, to calculate the phylogenetic distance between bacterial communities and to obtain the β value between samples (14). Unweighted Pair Group Method with Arithmetic Mean (UPGMA) was performed with the R language tool (version 3.6.2) to assess the similarity of bacterial composition between samples based on pairwise similarities. The clustering results were displayed using the web-based ETE Toolkit Phylogenetic tree (newick) viewer (v. 3.1.3) (https://etetoolkit.org/treeview/).

## RESULTS

The DNA purity ($A_{260}/A_{280}$) of the five metalworking fluid samples was relatively low, ranging from 1.03 to 1.39, whereas DNA concentrations varied from 12.50 to 80.50 µg/mL (date not shown). The DNA purity ($A_{260}/A_{280}$) of a soil sample used as a control was 1.45 using the same protocol. Despite low purity (an ideal $A_{260}/A_{280}$ ratio should be closer to 1.8), all DNA samples passed quality control at CD-Genomics and the 16S rRNA gene was sequenced for each sample. Between 73931 and 112822 raw reads were generated by sequencing (Table 2). The values of Q20 and Q30 in Table 3 show that read confidence overall was high, with values near or above 95%. The average length of the clean tags ranged from 408.94 to 429.45 nucleotides (Table 2). Out of the five metalworking fluid samples tested, the sample with the least

## FIGURE 1.

*Taxa abundance heatmap of samples based on standardized Z-scores. The Z-score calculates how many standard deviations a value is from the mean of its row. Color represents the abundance of genera, and the horizontal clustering indicates the similarity of the abundance of different genera between samples.*



confidence was from Reservoir 1B, which had a Q20 value of 98.58% and Q30 of 94.88%. The sample with the highest confidence was from Reservoir 3 with a Q20 of 99.04% and Q30 of 96.37% (Table 2).

A Z-score heatmap is a graphical representation of clustering using color gradients to represent the relative abundance of similar genera in a sample based on read counts. The Z-score calculates how many standard deviations a value is from the mean of its row. For example, a score of +2 indicates that the abundance of that genus at a given site is two standard deviations greater than the mean abundance of that genus across all sites (Figure 1). The heatmap results show that the relative abundance of bacterial genera varied significantly between samples (Figure 1). For example, *Mycobacterium* was very abundant in two of the five samples (Reservoir 2A and Reservoir 3) but less abundant in the other three samples, especially in Reservoirs 1A and 1B as shown by the blue color (Figure 1). Other genera, such as *Sphingobium*, have a high Z-score in sample from Reservoir 2B (Figure 1). On the other hand, *Pseudomonas* and *Acinetobacter* had high Z-scores in the Reservoir 1B sample indicating relatively high abundance levels in that sample (Figure 1). These data were further analyzed to tabulate the percentages of bacterial genera or families detected from each site based on read counts to visualize within-group comparisons (Table 3). Once again, *Mycobacterium* was very abundant in Reservoirs 2A and 3, representing 87.09% and 99.72% of the sequences, respectively (Table 3). *Mycobacterium* was also abundant at site 2B at 28.12% but was mostly absent from reservoirs 1A and 1B (Table 3). *Pseudomonas* (9.26%) and *Rhizobiaceae* (29.54%) were abundant at reservoir 2B (Table 3). Reservoir 1A mostly included bacteria from the genera *Comamonas* (64.80%) and *Elizabethkingia* (24.59%), whereas Reservoir 1B mostly consisted of *Acinetobacter* (76.45%) and *Pseudomonas* (18.66%) (Table 3).

Diversity indices such as Simpson and Shannon provide a measure of biodiversity by combining richness and evenness. Overall, samples from Plant 2 were found to have the greatest number of observed features (Operational Taxonomic Units)

## TABLE 3.

*The percentage relative abundance of the main bacterial genera or families detected in each metalworking fluid sample based on read counts.*

| Genus of Family | Percentage relative abundance (%) in each sample | | | | |
|---|---|---|---|---|---|
| | Reservoir 1A | Reservoir 1B | Reservoir 2A | Reservoir 2B | Reservoir 3 |
| *Acinetobacter* | -[a] | 76.45 | 1.23 | 5.29 | - |
| *Aerococcaceae* | - | - | 0.03 | 4.86 | - |
| *Aquamicrobium* | - | - | 0.07 | 1.18 | - |
| *Brevundimonas* | - | - | 0.78 | 0.03 | - |
| *Comamonas* | 64.80 | 0.01 | 0.12 | 2.47 | - |
| *Comamonadaceae* | 0.16 | - | 0.99 | 1.11 | - |
| *Dysgonomonas* | - | 1.34 | 0.03 | - | - |
| *Elizabethkingia* | 24.59 | - | - | - | - |
| *Methylorubrum* | 5.10 | - | - | - | - |
| *Microbacteriaceae* | - | - | 0.05 | 4.49 | 0.02 |
| *Mycobacterium* | 0.02 | - | 87.09 | 28.12 | 99.72 |
| *Paenochrobactrum* | - | - | 0.02 | 5.74 | - |
| *Phreatobacter* | 4.76 | - | - | - | - |
| *Pseudomonas* | 0.04 | 18.66 | 1.70 | 9.26 | - |
| *Rhizobiaceae* | - | 0.04 | 0.33 | 29.54 | 0.04 |
| *Sphingobium* | - | - | 1.44 | - | - |
| *Stenotrophomonas* | - | - | 0.32 | 1.71 | - |
| Others | 0.53 | 3.50 | 5.83 | 5.76 | 0.21 |

[a] Not detected

with Reservoir 2B having 114 different bacterial groups and Reservoir 2A having 367 groups (Table 4). Reservoir 2B had the highest diversity indices with a Simpson index of 0.909 and a Shannon index of 4.158. Table 3 also shows that Reservoir 2B had a relatively rich representation of different bacterial groups. As expected, Reservoir 3 had the lowest diversity indices with a Simpson index of 0.006 and a Shannon index of 0.040 (Table 4). This makes sense as the sample from Reservoir 3 was found to compromise 99.72% *Mycobacterium* (Table 3) and only 38 bacterial groups overall (Table 4). The other three samples had Shannon diversity indices ranging from 1.433 to 2.315 (Table 4).

Figure 2 shows species richness, diversity, and relatedness of the metalworking samples based on unweighted clustering (UPGMA). A UPGMA tree was constructed to visualize the differences among bacterial communities from the different reservoirs. A closer sample distance and a shorter branch indicate more similarity in bacterial composition between the samples. The large percentage of *Mycobacterium* in the sample from Reservoir 3 (Table 3), combined with its low diversity indices (Table 4), is the main reason it is the least related to any other sample. Samples collected from the same plants were seen to be phylogenetically related (Figure 2).

## DISCUSSION

In the present study, DNA was isolated and purified from five different metalworking fluid samples and successfully sequenced as part of a metagenomic analysis using the V3-V4 regions of the 16S rRNA gene. Overall, a large number of raw PE reads were generated and the Q30 for all samples was approximately 95% or higher. Our metagenomic study provides additional insights into the diverse bacterial communities present in automotive metalworking fluids and supports the notion that the presence of mycobacteria in some, but not all, metalworking fluids is significant.

As shown by the taxa abundance heatmap of samples, diversity indices, and clustering tree, there were significant differences between samples. The Shannon index for four of the five reservoirs ranged from 1.433 to 4.158, which is relatively similar to the Shannon index calculated (ranging from 1.86 to 2.93) in a metagenomic study conducted with spent metalworking fluids (8). Samples from the same plants (Reservoirs 1A and 1b; Reservoirs 2A and 2B) showed some relatedness between them, which is to be expected since similar products and environmental factors are present. Nevertheless, there were variations in bacterial communities between reservoirs as shown by the heatmap Z-scores which highlighted the relative abundance of certain genera at each site. For instance, Z-scores showed the relative abundance of mycobacteria in two of the five samples. The sample from Reservoir 3 yielded very low Simpson index (0.006) and Shannon index (0.040) in comparison to the other four samples. This might be due to the metalworking fluid at that site having been recently heavily treated with broad-spectrum sulfur containing antibacterial biocides (personal communication from the personnel), resulting in a fluid that contained very few types of bacteria. This would suggest that mycobacteria are especially resistant to this type of disinfection and would warrant additional evaluation. These results are similar to those of another study, in which relatively higher levels of *Mycobacterium* spp. were found in treated metalworking fluids than in untreated fluids (10). The current study is one of the first to use metagenomics to obtain a comprehensive analysis of bacterial communities

## FIGURE 2.

*UPGMA clustering tree based on unweighted UniFrac to visualize the relatedness of each individual metalworking fluid sample against one another. The support or bootstrap values are displayed at each node.*

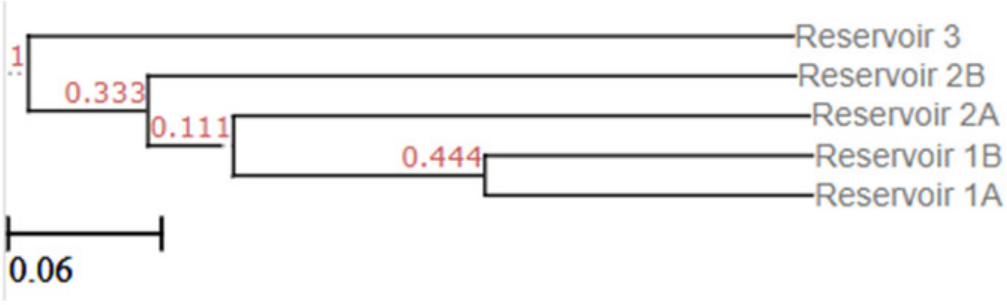


**TABLE 4.**

*Statistical analysis of alpha diversity indices in each metalworking fluid sample.*

| Sample | Bacterial taxa | Ace | Chao1 | Simpson | Shannon |
|---|---|---|---|---|---|
| Reservoir 1A | 102 | 102 | 102 | 0.516 | 1.433 |
| Reservoir 1B | 64 | 64 | 64 | 0.738 | 2.315 |
| Reservoir 2A | 367 | 367 | 367 | 0.241 | 1.444 |
| Reservoir 2B | 114 | 114 | 114 | 0.909 | 4.158 |
| Reservoir 3 | 38 | 38 | 38 | 0.006 | 0.040 |

in metalworking fluids. A recent Russian study used metagenomics to analyze metalworking fluids and identified a large number of bacterial and fungal genera including *Alcaligenes*, *Bacteroides*, and *Pseudomonas* (8). Two other studies, conducted in China and Switzerland, also used metagenomics to evaluate metalworking fluids from various industries (9, 10). Future metagenomic studies could provide additional insights into the microbiology of metalworking fluids in a variety of industries and under different manufacturing conditions. For example, evaluating changes in microbial communities with the use of specific biocides or disinfectants could be done using metagenomic analysis. Although metagenomics may not be appropriate for routine surveillance and monitoring of metalworking fluids, it is a powerful approach that may help address specific microbial contamination problems facing the automotive industry.

As in other studies (1, 8, 9, 10, 15, 16), many bacterial genera and families were detected in the five metalworking fluid samples analyzed in this study. *Mycobacterium* spp. and *Pseudomonas* spp. were found among the main identifiable bacterial groups in three of the five samples. The presence of *Pseudomonas* in metalworking fluids agrees with the results of other studies that have reported it as a predominant genus in metalworking fluids (8, 9, 10). At one site, Reservoir 3, *Mycobacterium* made up more than 99% of the bacterial composition, which warrants future research. The presence of mycobacteria is not surprising since *M. immunogenum* has been repeatedly found in metalworking fluids and is persistent in such systems. *Mycobacterium immunogenum* has been of increasing concern due to being linked to cases of hypersensitivity pneumonitis infections in industrial workers exposed to metalworking fluids (15). The detection of *Pseudomonas*, which is often associated with biofilms, in our bulk fluid samples suggests that sloughed-off biofilm cells are likely to contaminate metalworking fluids. Biofilm formation in metalworking fluid systems makes disinfection more challenging as biofilm bacteria are highly resistant to environmental stressors and chemical treatments (17). Therefore, the presence of *Mycobacterium* and *Pseudomonas* in biofilms could have implications on disinfection methods of metalworking fluid systems, as biofilms often remain even with the addition of biocides and general cleansing processes. In a 2008 study of a relatively small U.S. Midwest machining facility, *Alcaligenes faecalis* was identified from every sample collected during summer and winter sampling periods. However, *Mycobacterium immunogenum* was only detected from one machine in winter samples (22). In the present study, *Mycobacterium* was detected in samples collected during the fall, suggesting that future research should evaluate the effect of temperature and seasonality on the presence of mycobacteria in metalworking fluids. Additionally, *Alcaligenes* sp. was not found in any of our samples. Examples of other bacteria previously found in metalworking fluids, but not in this study, include *Bacteroides*, *Citrobacter*, *Legionella*, *Micrococcus* spp., *Propionibacterium* spp., *Pseudochrobactrum* spp., and *Staphylococcus capitis* (3, 8, 10, 16).

Several other bacterial groups were identified in our samples. The dominant taxonomic units vary, which is probably due to the unique conditions of each reservoir. For example, *Rhizobiaceae*, a family of soil bacteria, is most notable for its nitrogen fixing symbiotic relationship with plant roots (18). Its high presence in Reservoir 2B (at more than 29% of its overall composition) may indicate soil or sand contamination because the rough automotive parts machined using the metalworking fluid from Reservoir 2B come from a casting plant that uses sand and iron filings in the casting molds. Several large operations take place in Reservoir 2B, which may release some sand. This is not necessarily the case with other reservoirs. Both *Comamonas* and *Elizabethkingia* are genera of bacteria common to the environment and both with documentation of occasional virulence to humans (19). The level of *Comamonas* was especially high (almost 67%

of bacteria) in Reservoir 1A. Species of this genus are found not only in the natural environment but also in engineered conditions such as factory settings, where they play a role in nitrate reduction (19). *Comamonas* was also very abundant in metalworking fluids used for cutting aerospace materials at a research institute in Shanghai, China (9). *Elizabethkingia* is a genus commonly found in both the environment and notably, in hospital tap water. Historically, *Elizabethkingia* species have caused illness in immunocompromised patients. The most important of these is *E. meningoseptica*, which was responsible for a meningitis outbreak in the 1950s (20). The presence of both *Comamonas* and *Elizabethkingia* in Reservoir 1A indicates a need for further research to determine whether known pathogenic species are present. *Acinetobacter* spp. was found at a high percentage in Reservoir 1B (more than 76%) and to a lesser extent (more than 5%) in Reservoir 2B. Its presence is not surprising; *Acinetobacter* was first detected in metalworking fluids in 1987 and since then has been shown to be a very common contaminant of metalworking fluids (9, 21, 22). It is an opportunistic pathogen that can form biofilms and cause a variety of infections in blood, lungs, or wounds (23).

The results of this study indicate that our DNA isolation procedure works well to obtain metagenomically analyzable DNA. However, a different metagenomic analysis (for example, full-length 16S rRNA sequencing) could be done to identify species and subspecies of bacteria. In general, a wide variety of taxonomic groups were found at each site with the exception of Reservoir 3. As in several other studies on metalworking fluids, all samples were found to have bacterial communities that include opportunistic pathogens (e.g., *Mycobacterium, Pseudomonas, Acinetobacter*), making further surveillance of metalworking fluids important to closely monitor pathogenic bacteria. The high percentages of *Mycobacterium, Acinetobacter*, and *Pseudomonas* in samples indicate that there may be biofilms composed of those bacteria present in metalworking fluids, making further research of the biofilms of interest, specifically at the species level. A limitation of this type of study, however, is that DNA detection does not necessarily imply that viable cells are present in a sample.

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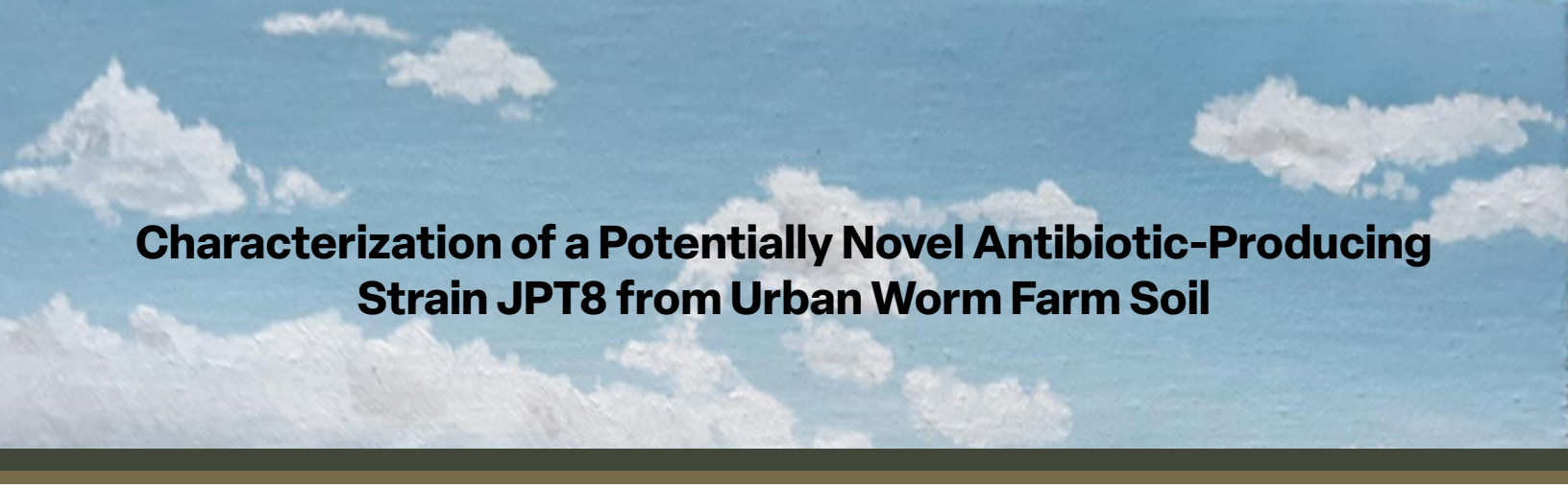

# Characterization of a Potentially Novel Antibiotic-Producing Strain JPT8 from Urban Worm Farm Soil

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## ABSTRACT

Antimicrobial resistance (AMR) poses an escalating global health crisis, with few novel structural classes of antibiotics approved in decades. To address this gap, we isolated and characterized a potentially novel antibiotic-producing bacterium, strain JPT8, from urban worm farm soil – an underexplored niche enriched by organic inputs and anthropogenic selective pressures – using a multidisciplinary pipeline combining microbiology, biochemistry, and molecular biology.

Serial dilution and plating yielded over 500 colonies. Four isolates showed reproducible inhibition against *Staphylococcus aureus* in patch-on-lawn assays, with JPT8 producing the largest zones (up to 23 mm). Scale-up fermentation in minimal media, dichloromethane extraction, and bioactivity-guided fractionation via thin-layer and silica gel column chromatography identified stable bioactive compounds in fractions S7 and S10 (10–20 mm zones in disc diffusion). Morphological (circular, undulate, tan colonies), biochemical (Gram-positive, catalase-positive, endospore-forming), and 16S rRNA gene sequencing (forward read: 1129 bp raw, trimmed to high-confidence 616 bp alignment with 88% identity) revealed only 82% pairwise similarity to the closest match (*Bacillus pumilus* NJ-M2, NZ_CP012329.1) and 79% to other *Bacillus* references, falling well below standard species delineation thresholds (~98.7%).

These findings suggest that JPT8 represents a divergent lineage with antimicrobial potential; however, further genomic and chemical characterization is required.

**Keywords:** AMR, *Bacillus*, bioactive compounds, 16S rRNA sequencing, PCR, urban worm farm soil, vermicompost, microbiome.

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## INTRODUCTION

Antimicrobial resistance (AMR) represents one of the most pressing global health threats, rendering one in six bacterial infections resistant worldwide (1). This trend, driven by overuse in clinical, agricultural, and environmental settings, has compromised frontline treatments for common infections and increased mortality risks from multidrug-resistant bacterial infections, including both Gram-positive and Gram-negative pathogens. Despite the urgency, antibiotic discovery pipelines have largely stalled: no new structural classes have reached clinical use in over three decades, and most recent approvals are derivatives of existing scaffolds that rapidly encounter resistance (2,3). The decline stems from high attrition rates in traditional screening from known microbial sources, low hit rates against resistant strains, and economic barriers to development. Soil microbiomes remain a rich source of bioactive natural products, although conventional sampling has yielded diminishing returns (4,5). Underexplored niches, such as urban worm farm soils, may provide access to distinct microbial communities with antimicrobial potential (6,7).

This study aimed to isolate and characterize antibiotic-producing bacteria from urban worm farm soil. The environment was hypothesized to harbor microorganisms with bioactive potential against pathogens such as *Staphylococcus aureus*, which could be identified through an integrated microbiological, biochemical, and molecular pipeline. This approach led to the identification of strain JPT8, which exhibited potent activity against *S. aureus* and partial sequence novelty relative to known Bacillus species. Subsequent fractionation confirmed the presence of bioactive components, laying the foundation for advanced structural elucidation. These findings highlight urban soil microbiomes as an accessible, high-yield source for antibiotic discovery and demonstrate how streamlined multidisciplinary methods can revive natural product pipelines amid escalating AMR (8).

## MATERIAL AND METHODS

The experimental workflow (Figure 1) began with sample collection and bacterial isolation, followed by primary antimicrobial screening using patch-on-lawn assays. After the potent isolate JPT8 was identified in the initial screening, two parallel workflows were pursued: (1) Scale-up fermentation, biomolecular extraction, and chromatographic fractionation for bioactivity guided purification of active compounds, and (2) morphological, biochemical, and molecular characterization of the strain.

### REAGENTS AND MATERIALS

The reagents used in this study, along with their suppliers, were as follow: Trypticase soy agar (TSA), nutrient agar (NA), nutrient broth (NB), and Mueller-Hinton agar (MHA) from Carolina® Biological (Burlington, NC, USA); minimal media components including soluble starch, yeast extract, peptone from Carolina® Biological (Burlington, NC, USA); Dichloromethane, methanol, chloroform, diethyl ether, toluene, ethyl acetate, and Physiological saline (0.85% NaCl) from Fisher Scientific (Pittsburgh, PA, USA) and Acros Organics (New Jersey, USA); DNeasy Blood and Tissue Kit for DNA extraction from Qiagen (Hilden, Germany); universal 16S rRNA primers (27F and 1492R) – Integrated DNA Technologies (San Diego, CA, USA); silica plates (Whatman 4410222); and sterile 6 mm disc (Becton Dickinson BBL, Sparks, MD, USA).

### SAMPLE COLLECTION AND BACTERIAL ISOLATION

Soil samples were collected from an urban worm farm at 10–20 cm depth using a sterile borer in December 2014 and stored at 4°C. One (1) gram of soil was suspended in 9 mL sterile physiological saline (Fisher Scientific), homogenized by vortexing for 1 min, and serially diluted to $10^2$, $10^3$, $10^4$, $10^5$, and $10^6$ concentrations. Aliquots (0.1 mL) were spread on trypticase soy agar (TSA; Carolina 788428) and incubated at 28°C for 3 days to achieve proper growth. Distinct colonies were purified by repeated streaking on nutrient agar (NA; Carolina 785301) at 28°C for 24 h and preserved at 4°C for further experimentation.

### ANTIMICROBIAL SCREENING

Physiologically distinct microbial populations were selected for study of their antimicrobial properties. Gram-positive bacteria (*Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*) were activated in nutrient broth (NB Carolina 785361) for 18 h at 37°C before careening. Primary screening used patch-on-lawn assays on Mueller-Hinton agar (MHA; Carolina 784942) at 37°C for 24 h. The patches were placed on fresh lawn cultures of bacterial strains, and the plates were incubated at 37°C for 24 h. The secondary screening of extracted biomolecules employed the Kirby-Bauer disc diffusion assay. A known amount of extract was absorbed onto 6-mm sterile discs (BD, BBL), and the discs were placed on fresh lawn growth of test organisms on MHA media. All experimental plates were incubated at 37°C for 24 h. To ensure the accuracy and objectivity of inhibition zone measurements, a standardized protocol was followed. All zones were measured manually using a calibrated metric ruler against a high-con-

trast background to the nearest 0.1 mm. For disc and patch, two perpendicular diameters were measured, and the average value was recorded.

### SCALE-UP AND BIOMOLECULE EXTRACTION

Potent culture - JPT8 was selected for mass culturing using lab scale fermentation. A well-isolated colony was inoculated in minimal media containing 1% starch, 0.4% yeast extract, 0.2% peptone and incubated for 7 days with shaking. The biomass was harvested by centrifugation, and metabolites were extracted with an equal volume of dichloromethane (DCM) in duplicate. Extracts were evaporated under reduced pressure using a rotary evaporator (Buchi), air-dried, and collected as vial 1 and vial 2, respectively. The dried extract was resuspended in dichloromethane to a concentration of 10 mg/mL and absorbed onto sterile 6 mm disc (BD, BBL) for disc diffusion testing.

### CHROMATOGRAPHIC FRACTIONATION

Crude extracts were analyzed by thin-layer chromatography (TLC) on silica plates (Whatman 4410222) developed in methanol: chloroform (9:1) and visualized under UV light. Preparative fractionation used silica gel column chromatography with diethyl ether: toluene gradient ratio: 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1. Ten fractions (S1 – S10) were collected in sterile screw cap vials, evaporated, and screened for antimicrobial activity.

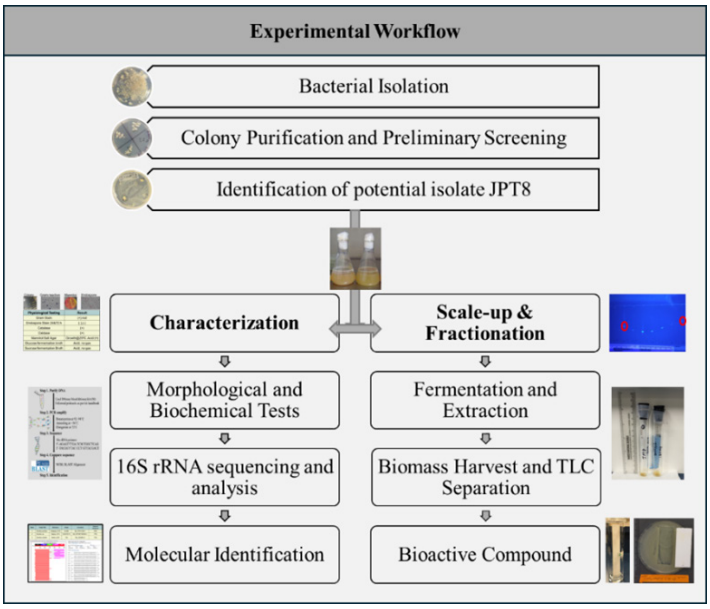
### PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF STRAIN JPT8

Strain JPT8 was characterized phenotypically by colony morphology on nutrient agar, Gram staining, endospore formation, catalase and oxidase tests, and sugar fermentation patterns according to established protocols (9).

For molecular characterization, JPT8 was cultured in minimal broth for 72 h and harvested by centrifugation at 5000 rpm for 10 min. The supernatant was discarded, and the cell pellets were resuspended in 180 µL of enzymatic lysis buffer, then incubated at 37ºC for 30 min. Subsequently, 25 µL proteinase K and 25 µL Buffer AL were added, mixed by vortexing, and incubated at 56ºC for 30 min. Genomic DNA was purified using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol. The lysate was applied to a DNeasy spin column and centrifuged at 8000 rpm for 1 min. The column was washed sequentially with 500 µL Buffer AW1 and 500 µL Buffer AW2, followed by centrifugation at 8000 rpm for 1 min and 20,000 rpm for 3 min, respectively.

## FIGURE 1.

*Workflow for isolation, characterization, and bioactivity-guided analysis of strain JPT8.*




The schematic illustrates bacterial isolation and preliminary screening, followed by parallel scaleup pathways for strain characterization (morphological, biochemical, and 16S rRNA analysis) and fermentationbased extraction with chromatographic separation of bioactive compounds.

The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using universal bacterial primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The resulting 16S rRNA gene amplicon from strain JPT8 was sequenced in the forward direction only using primer 27F. The resulting Sanger read (raw length 1129 bp, containing 360 ambiguous 'N' bases, which reduced the usable high-quality sequence length) was quality-trimmed (Phred ≥20) and analyzed using BLASTN against the NCBI nucleotide database.

All experiments were performed in triplicate. Results are reported as mean ± standard deviation (n=3). No statistical comparisons between groups were performed.

## RESULTS

### ISOLATION OF ANTIBIOTIC-PRODUCING STRAINS

Serial dilution and plating on trypticase soy agar yielded over 500 colonies after 3 days at 28°C. Eleven morphologically distinct isolates were purified by repeated streaking on nutrient agar. Patch-on-lawn assays against *Staphylococcus*

*aureus* identified four strains, whereas *Escherichia coli* showed no recordable zone of inhibition. Strain JPT8 produced the largest inhibition zones (22.6 ± 1.06 mm, n = 3) in initial patch assays and was selected for further characterization as the most promising candidate (Figure 2a/2b).

## ANTIMICROBIAL ACTIVITY AND BIOMOLECULE FRACTIONATION

Scale-up fermentation of JPT8 produced crude dichloromethane extracts with consistent activity against *S. aureus* yielding a mean inhibition zone of 17.4 ± 2.12 mm (Vial 1) and 23.5 ± 1.17 mm (Vial 2) (n = 3) in disc diffusion assays (Figure 2c). Thin-layer chromatography (TLC) of crude extracts in Methanol: Chloroform (9:1) resolved four UV-visible bands, indicating chemical complexity. The extract was separated into 10 fractions by silica gel chromatography. Fractions S7 and S10 retained strong antimicrobial activity with mean inhibition zone 10.4 ± 0.72 mm and 19.5 ± 0.70 mm (n = 3), respectively (Figure 2d). These results suggest that JPT8 produces one or more bioactive compounds with stronger activity against *S. aureus* than against *E. coli* amenable to bioactivity-guided fractionation.

## MOLECULAR IDENTIFICATION AND CHARACTERIZATION OF STRAIN JPT8

Morphological and biochemical tests indicated JPT8 as a Gram-positive, endospore-forming rod. Colonies on nutrient agar were circular, approximately 2 mm in diameter, with undulate margins, opaque texture, dryness, and tan coloration. The strain was catalase-positive, oxidase-negative, and fermented sugars. These traits are consistent with the genus *Bacillus*.

BLASTN analysis yielded a local alignment of 616 bp with 88% identity (541/616 matches, 5 gaps) to the 16S rRNA gene region in the *Bacillus pumilus* NJ-M2 genome (NZ_CP012329.1, positions 91033–91646). Overall pairwise similarity across the query was 82% to *Bacillus pumilus* NJ-M2, 79% to *Bacillus* sp. EGD-AK10, and 79% to *Bacillus subtilis* 168. This local alignment length reflects the high-quality, trimmed portion of the forward Sanger read after removal of low-confidence bases and ambiguities (N's). This phylogenetic positioning is illustrated in Figure 3.

## DISCUSSION

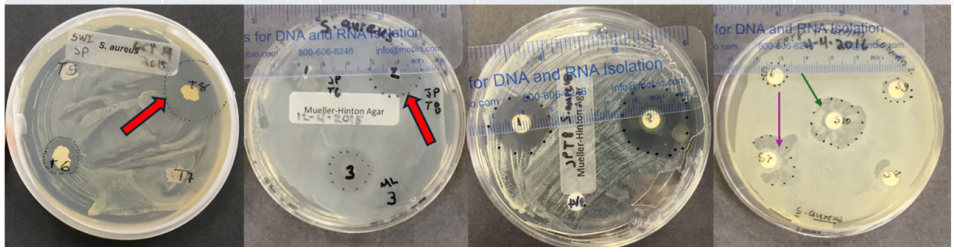
The isolation of strain JPT8 from urban worm farm soil yielded a Gram-positive bacterium with consistent antimicrobial activity against *Staphylococcus aureus* but none against *Escherichia coli.* This Gram-positive specificity is likely due to the outer membrane barrier in Gram-negative bacteria, which restricts access of the non-polar bioactive compounds to intracellular targets. Such selectively could be advantageous for targeting therapies against pathogens such as methicillin-resistant *S. aureus* (MRSA) without broadly disrupting commensal Gram-negative microbiota.

Morphological and biochemical tests confirmed traits consistent with the genus *Bacillus.* However, 16S rRNA sequencing provides overall pairwise similarities well below ~98.7% threshold typically required for species delineation and indicates phylogenetic divergence within the genus. Whole-genome sequencing or multi-locus sequencing will be

## FIGURE 2.

*Antimicrobial efficacy of strain JPT8: Antimicrobial efficacy of strain JPT8 against* Staphylococcus aureus.

| Original Culture | | Crud DCM Extract | | Column Purified Fraction | |
|---|---|---|---|---|---|
| JPT8 Patch 1 | JPT8 Patch 2 | Vial 1 | Vial 2 | Fraction S7 | Fraction S10 |
| 22.6 ± 1.06 mm | 20.5 ± 0.86 mm | 17.4 ± 2.12 mm | 23.5 ± 1.17 mm | 10.4 ± 0.72 mm | 19.5 ± 0.70 mm |




This multi-panel figure demonstrates the inhibitory activity of JPT8 extracts using patch-on-lawn and disc diffusion assays. All values are mean ± standard deviation from triplicate measurements (n=3). (Panels A–D correspond to original patch, purified colony, crude extract Vial 1/2, and purified fractions S7/S10, respectively.)

necessary to confirm whether JPT8 represents a novel species or a highly divergent lineage.

Bioactivity-guided fractionation via TLC and silica gel column chromatography produced stable active fractions (S7 and S10) that retained strong antimicrobial activity against *S. aureus*. A notable limitation of the extraction protocol is the exclusive use of dichloromethane, a non-polar solvent that preferentially recovers lipophilic compounds. Because no parallel aqueous or methanolic extractions were performed, highly polar bioactive metabolites may have been missed. Antimicrobial activity was limited to two model organisms (*S. aureus* and *E. coli*) which restricting insights into the full antimicrobial spectrum and the mechanism of action.

Urban worm farm (vermicompost) soil represents an underexplored anthropogenic niche that may favor microbes with competitive bioactive traits. The low-resource, multidisciplinary pipeline demonstrated here offers a scalable model suitable for an educational setting.

## CONCLUSION

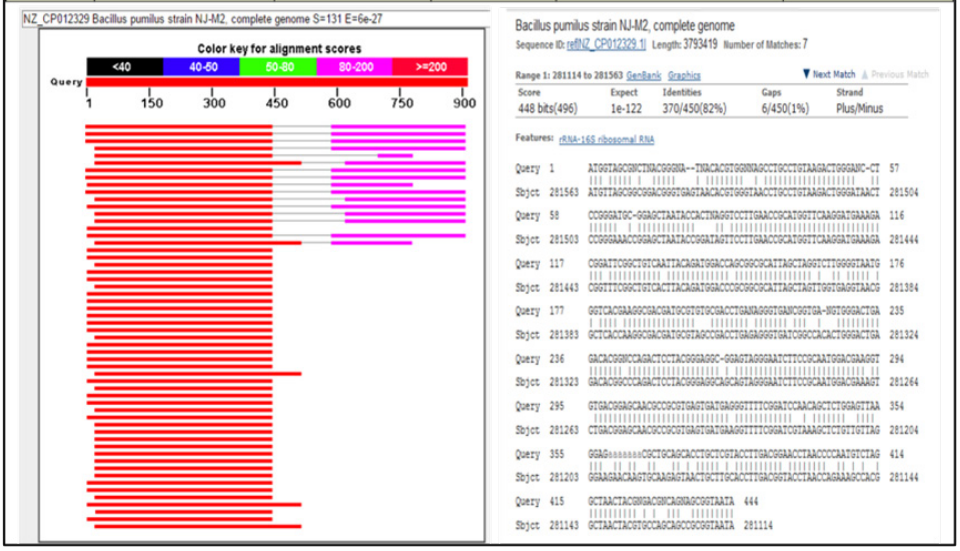
In summary, strain JPT8 isolated from urban worm farm soil demonstrated reproducible Gram-positive-specific antimicrobial activity against *Staphylococcus aureus* (zone of inhibition 17–23 mm) and 16S rRNA sequencing divergence from known *Bacillus* species (82% pairwise similarity). These findings supported by microbiology, biochemistry, and bioactivity-guided fractionation data, position JPT8 as a potential lead for further antibiotic discovery.

The multidisciplinary integration proved to be an effective, scalable approach to low-cost and accessible methods. Future work should focus on whole-genome sequencing of JPT8 to resolve its taxonomic position and identify biosynthetic gene clusters, structural elucidation of the active fractions (S7 and S10) using LC-MS/NMR, and expanded antimicrobial testing against clinically relevant resistant pathogens, including MRSA. These steps will determine the true potential of this early-stage discovery in contributing to the fight against antimicrobial resistance.

## FIGURE 3.

*16S rRNA-based similarity of strain JPT8 to closest* Bacillus *relatives. JPT8 shows 82% pairwise similarity to* B. pumilus *NJ-M2 (NZ_CP012329.1), 79% to* Bacillus *sp. EGD-AK10 (NZ_AVPM01000844.1), and 79% to B. subtilis 168 (NC_000964.3).*

| Rank | Name/Title | Reference | Strain | Accession | Pairwise Similarity |
|---|---|---|---|---|---|
| 1 | *Bacillus pumilus* | Weigang 2015 | NJ-M2 | NZ_CP012329.1 | 82% |
| 2 | *Bacillus sp* | Kaplay, 2013 | EGD-AK10 | NZ_AVPM01000844.1 | 79% |
| 3 | *Bacillus subtilis* | Barbe, 2009 | 168 | NC_000964.3 | 79% |




These results, combined with the morphological, biochemical, molecular identity, and Gram-positive-species specific antimicrobial activity, support the interpretation of substantial phylogenetic divergence and potential taxonomic as well as antimicrobial novelty for JPT8.

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# Novel Antibiotics Approved by the FDA (2022–2025): Mechanisms, Applications, and Clinical Relevance

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## ABSTRACT

Antimicrobial resistance (AMR) is a growing global health crisis, emphasizing the urgent need for new antibiotics. Despite high costs and long development timelines, several novel antibiotics were approved by the U.S. Food and Drug Administration (FDA) between 2022 and 2025. This review summarizes the antibiotic discovery process, the FDA approval pipeline, and highlights key novel agents including Voquezna, Xacduro, Orlynvah, Zevtera, Exblifep, and Blujepa. These therapies employ diverse strategies to achieve selective toxicity, such as inhibition of bacterial cell wall synthesis, β-lactamase activity, and DNA replication, as well as modulation of host-pathogen environments, while limiting damage to host tissues. This review highlights their mechanisms of action, approved clinical indications, and safety profiles, and evaluates their clinical relevance in treatment of infections caused by multidrug-resistant pathogens. Lastly, we discuss the implications of these recent approvals for the future of antibiotic development and emphasize the continued need for sustained investment and innovation to address the growing AMR crisis.

**Keywords**: Antimicrobial resistance (AMR), FDA approval, Drug development, Antibiotic development, Artificial intelligence (AI)

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## INTRODUCTION – THE URGENT NEED FOR NOVEL ANTIBIOTICS:

Antimicrobial resistance (AMR) is a major global health problem that makes antibiotics less effective against infections. Almost every variant of human pathogenic bacteria has developed resistance to or become less susceptible to current antibiotic treatments (10). The excessive and improper use of antibiotics in healthcare and agriculture has contributed to the rapid emergence and spread of antibiotic-resistant bacteria (54). According to the CDC, the U.S. experiences over 2.8 million antimicrobial-resistant infections each year, leading to over 35,000 deaths (3). Globally, antibiotic-resistant bacteria are responsible for over 700,000 deaths annually (11). Without major advances in antibiotic development, deaths from resistant infections could reach 10 million annually by 2050 (5).

Epidemiological studies show that antibiotic resistance places a major economic burden, causing around 11 million extra hospital stays and more than $20 billion in additional healthcare and social costs each year in Europe and the United States (15), (20). Therefore, reducing the unnecessary use of antibiotics is crucial for preserving the effectiveness of existing drugs, while strengthening research and development of new medications is also essential to address the growing problem of antibiotic resistance (10).

The rise in bacterial resistance calls for global cooperation among governments, pharmaceutical companies, biotech firms, and academic institutions to develop new antibacterial drugs (24). In this effort, researchers are focusing on creating novel, powerful, long-lasting, and broad-spectrum antimicrobial agents from diverse sources (8), (16). While antibiotic development has slowed, there have still been notable advancements, such as the discovery of new antibiotics, the revival of older treatments, and the creation of new semi-synthetic forms (24).

## MODERN ANTIBIOTIC DISCOVERY:

Worldwide, groups of scientists are taking different approaches for finding antibiotic compounds. One approach involves evaluating bacteria for their ability to make antimicrobial compounds. A recent paper identified a *Streptomyces thinghirensis* strain called MSA1 with antibacterial properties against multiple significant bacterial pathogens (21). This was done through screening bacterial isolates for their ability to inhibit the growth of prevalent pathogens like *S. aureus* and *P. aeruginosa*. They then went on to optimize the conditions that would cause MSA1 to produce the antimicrobial compound and assessed the necessary concentrations to inhibit growth of various pathogens. The group did not identify what this compound was chemically.

Another approach identifies chemical compounds directly through *in silico* screening. A recent paper evaluated potential small molecule drug targets for $LcpA_{SA}$, which is a member of the LytR-CpsA-Psr (LCP) family of enzymes in *S. aureus* (27). This protein is part of the cell membrane and essential for the synthesis of the cell wall (56). The LCP family of enzymes have potential to be a strong therapeutic target. The researchers virtually screened libraries of chemical compounds to look at compounds that had strong binding affinity for $LcpA_{SA}$ and that could act as inhibitors (27). Through further screening they identified seven compounds that bound effectively. These compounds also possessed characteristics that would make them effective drugs. The study did not test the compounds *in vitro* or *in vivo*.

Despite research like the examples above, conventional antibiotic development has significantly slowed in recent decades due to the high financial burden, lengthy timelines averaging over a decade, and low success rates of candidate molecules (20). In response, artificial intelligence (AI) technologies, particularly machine learning (ML) and deep learning (DL), have emerged as powerful tools to revitalize the antibiotic discovery pipeline (20), (29). Various AI approaches have demonstrated significant potential in accelerating the identification of novel antimicrobial agents. For example, ML combined with high-throughput Fourier-transform infrared spectroscopy successfully predicted antibiotic mechanisms of action and potency, while random forest models were used to assess phenotypic changes and predict antibacterial activity (50), (71). Deep learning and neural networks identified halicin, a compound with potent activity against drug-resistant pathogens (62). Similarly, ML algorithms have been used to identify β-lactamase inhibitors, predict and classify bacteriocins, and design bacteriocin-derived peptides with significant antimicrobial activity (45), (56), (71). These applications underscore the versatility and efficiency of AI in reducing the time, cost, and uncertainty associated with early-stage antibiotic development, offering a promising path forward in addressing the growing crisis of antimicrobial resistance (15).

From these examples, it is clear that there are many technologies and techniques that can be applied to discovering novel antibiotics, which must undergo rigorous testing before FDA approval for human use.

## DRUG DEVELOPMENT AND APPROVAL BY THE FDA:

Developing a drug and getting it to market is costly. On average, this process can cost around $800 million, covering expenses related to research, development, and clinical trials (14). Turning a promising antibiotic into a product that patients can use is a long and difficult process, often taking over ten years from discovery to final approval (14). The process begins with discovery and preclinical studies assessing antimicrobial activity, toxicity, and pharmacokinetics, followed by phased clinical trials to evaluate safety and efficacy in humans. Phase I trials focus on safety and dosing in healthy volunteers, phase II trials assess preliminary efficacy and optimal dosing in patients, and phase III trials confirm clinical effectiveness and monitor adverse effects in larger patient populations (66).

If clinical trials are successful, the drug sponsor submits a New Drug Application (NDA) to the FDA, initiating a comprehensive review process that typically takes one to two years. During this period, the FDA evaluates the drug's safety, clinical efficacy, manufacturing quality, pharmacokinetics, and benefit-risk profile, as well as compliance with Good Manufacturing Practices (GMP) and labeling requirements (41), (66). Following approval, post-marketing (Phase IV) studies continue to monitor long-term safety, effectiveness, and the emergence of rare adverse events (63).

Together, this regulatory framework shapes which antibiotic candidates ultimately reach clinical use and provides important context for understanding the limited number of antibiotics approved each year.

## REVIEW OBJECTIVES:

The rigorous research, development, and testing process required for FDA approval is necessary to ensure that only safe and effective drugs can be used for treating patients. With the high cost of developing drugs, pharmaceutical companies tend to place resources that will result in the most profits. For this reason, novel antibiotics are not as high priority as drugs that would be used long-term. This results in low amounts of antibiotics that are FDA approved each year compared to other drug types. This review aims to discuss antibiotic treatments that have not been previously approved in the United States. At the same time, we aim to bring awareness to the critical need for the development of novel antibiotics.

## NOVEL ANTIBIOTICS APPROVED BETWEEN 2022-2025

### VOQUEZNA AS A COMBINATION TREATMENT FOR *H. PYLORI*

Voquezna Triple Pak contains three drugs, Vonoprazan, amoxicillin, and clarithromycin to serve as a combination treatment for *Helicobacter pylori* infections in adults (42). It was approved by the FDA on May 3, 2022. Voquezna is taken orally in a capsule or tablet with 500 mg amoxicillin, 500 mg clarithromycin, and 20 mg Vonoprazan fumarate. There is also the Voquezna Dual Pak which contains only Vonoprazan and amoxicillin that was approved alongside the Triple Pak. Vonoprazan itself is not an antibiotic but is part of a combination regimen with amoxicillin only for the Dual Pak and also clarithromycin for the Triple Pak. Amoxicillin inhibits bacterial cell wall synthesis and clarithromycin binds the 50S ribosome inhibiting protein synthesis (2), (46). These antibiotics are not newly approved drugs, so we will focus on the function of Vonoprazan and how it improves the efficacy of the antibiotics it is dosed with.

Vonoprazan is part of a class of drugs known as potassium-competitive acid blockers (PCAB) and is the only member of its class currently approved for use in the United States (55). Vonoprazan is able to reversibly bind to the $H^+$-$K^+$-ATPase pump in the stomach (Figure 1) (36). This pump is essential for the secretion of stomach acid. When Vonoprazan binds to this pump, acid secretion is reduced because the $H^+$-$K^+$-ATPase enzyme is inactivated. This is beneficial for treating *H. pylori* infections.

At a gastric pH of approximately 6-7 *H. pylori* actively replicates and is susceptible to antibiotic treatment, whereas at lower pH values (approximately 3-6) the bacterium transitions into a coccoid, dormant form that is largely resistant to antimicrobial therapy (31), (32). Vonoprazan increases gastric pH by inhibiting acid secretion, which keeps *H. pylori* in its replicative, antibiotic-susceptible state and enhances the effectiveness of co-administered agents such as amoxicillin and clarithromycin (Figure 1). The selective toxicity is achieved indirectly through host gastric acid modulation, which shifts *H. pylori* into its replicative, antibiotic-susceptible state while minimizing direct toxicity to host tissues.

The traditional standard of care for *H. pylori* infection involves treatment with a proton pump inhibitor (PPI) (31). This method has lost effectiveness over time and takes about three days to effectively and stably suppress the secretion of gastric acid (69). Vonoprazan effectively blocks acid secretion within the first day of use (37). A recent meta-analysis of

Vonoprazan dual or triple regimen clinical data from multiple western and eastern countries showed that overall efficacy of 86% and 88%, respectively (7). The same study also determined that overall efficacy of treating *H. pylori* infections in treatment naïve patients was increased when using regimens with Vonoprazan compared to PPIs (87% vs 70%).

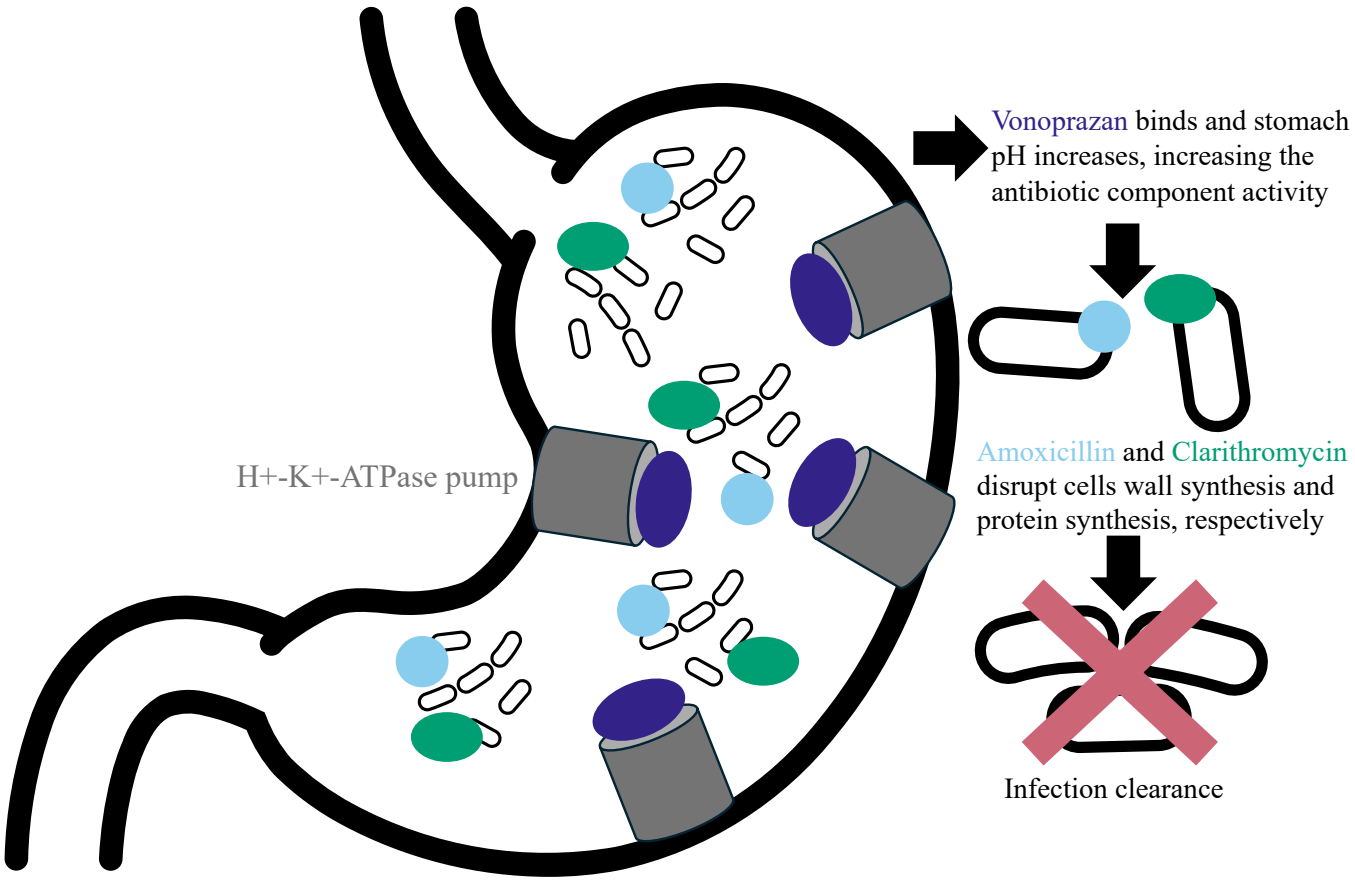
Vonoprazan is considered safe when compared to other treatments for *H. pylori* infection (69). When comparing the safety of a Vonoprazan and amoxicillin regimen to a PPI and amoxicillin regimen this study found that adverse reactions were significantly lower for the group treated with Vonoprazan and amoxicillin. Compliance for the treatment was also significantly higher for the Vonoprazan and amoxicillin treatment group.

While they are considered safe, both the triple Pak and dual Pak treatments can cause unintended side effects. The Vonoprazan-associated side effects include abdominal pain, abdominal bloating, diarrhea, headache, and dysgeusia (36). These side effects are common among antibiotic-containing treatment regimens. Upon treatment termination, these side effects are resolved. There is a current gap in our understanding of long-term adverse side effects of Vonoprazan use. In the context of its use of improving antibiotic function, long-term use should not be relevant since Voquezna is not a drug meant for long-term use.

Voquezna is an example of using new drugs to amplify the efficacy of existing antibiotics. Without the Vonoprazan component, both amoxicillin and clarithromycin would likely be less effective due to the biology of *H. pylori*. It is possible that in the future new PCABs could be developed that target

## FIGURE 1

*Voquezna Triple Pak mechanism of action.*




Voquezna consists of Vonoprozan, amoxicillin, and clarithromycin used for the treatment of *Helicobacter pylori*. Vonoprozan is a new class of drug known as a potassium-competitive acid blocker (PCAB). Vonoprozan reversibly binds to the H+-K+-ATPase pump in the stomach. This results in a pH increase within the stomach causing *H. pylori* to be more susceptible to the well-established antibiotics, amoxicillin and clarithromycin also found within Voquezna. Amoxicillin disrupts cell wall synthesis and clarithromycin disrupts protein synthesis, clearing the *H. pylori* infection.

other cell-specific $H^+$-$K^+$-ATPase along the GI tract that could impact the microbiota or help clear infections of the GI tract.

## XACDURO AS A TREATMENT FOR PNEUMONIA CAUSED BY *ACINETOBACTER BAUMANNII-CALCOACETICUS* COMPLEX

Xacduro is approved for administration intravenously for the treatment of hospital- and ventilator-acquired bacterial pneumonia (42). Xacduro is a combination drug containing Sulbactam and Durlobactam. It was approved by the FDA on May 23, 2023. Xacduro is dosed over the course of three hours as 1.0 g of Sulbactam and 1.0 g of Durlobactam. While this combination is considered to have a broad spectrum of susceptible bacteria, it should only be used to treat strains of *Acinetobacter baumannii-calcoaceticus* (ABC) that are shown to be susceptible to the drug (33), (42). For non-ABC infections, use of Xacduro should be guided by susceptibility testing, as its approval and demonstrated efficacy are limited to ABC pathogen.

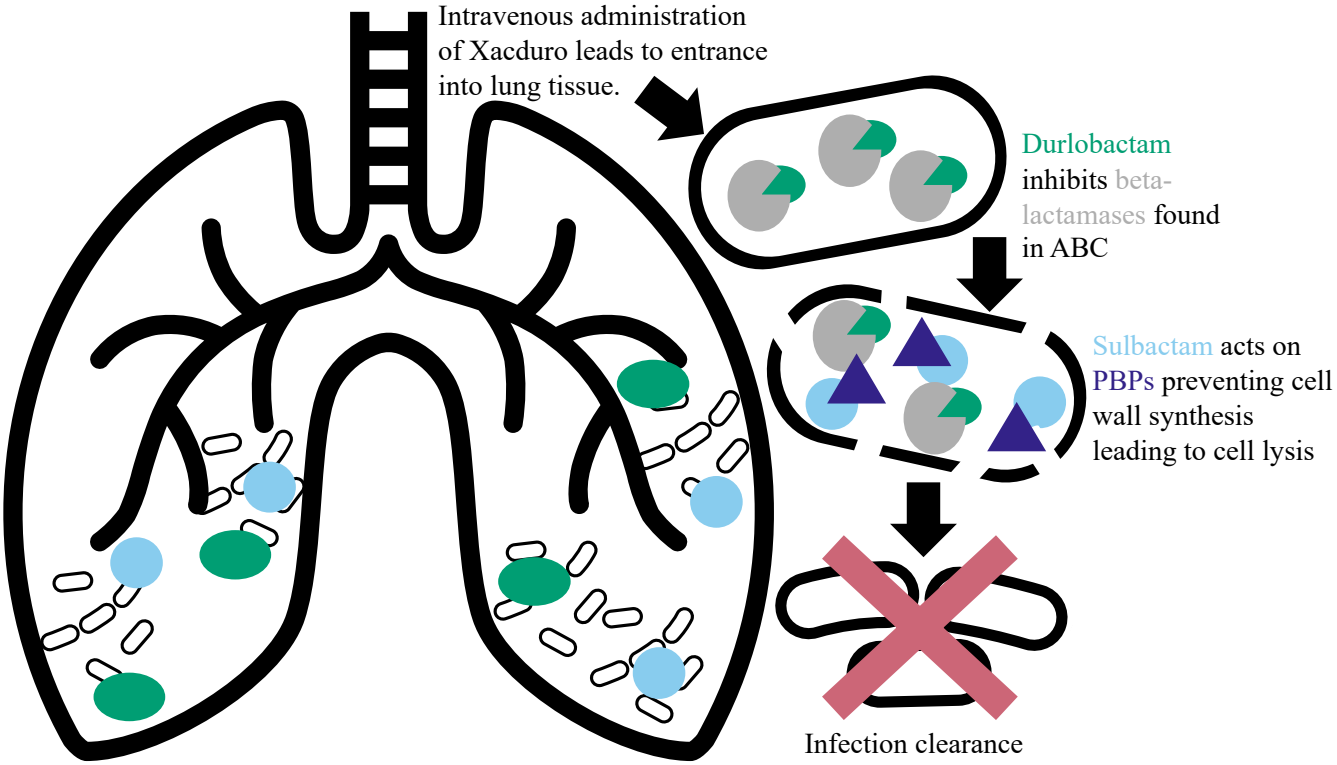
Sulbactam has been used in the past in combination with ampicillin to treat a variety of infections (9). These infections include skin and skin structure infections, intra-abdominal infections, and as an alternative treatment for gynecological infections caused by *Neisseria gonorrhoeae* or *Chlamydia trachomatis*. The other bacteria found to be susceptible to Sulbactam in combination with ampicillin include *Haemophilus influenzae*, *Escherichia coli*, *Acinetobacter*, *Klebsiella*, *Staphylococcus aureus*, *Enterobacter*, and anaerobes.

Sulbactam is a first generation β-lactam-β-lactamase inhibitor that resembles penicillin (33). It can inhibit Ambler class A serine beta-lactamases (1), (18). This allows it to improve the function of other β-lactam antibiotics that could otherwise be degraded by β-lactamase producing pathogens. In combination with amoxicillin, Sulbactam serves as a β-lactamase inhibitor to allow for amoxicillin to be effective.

ABC was once directly susceptible to Sulbactam. Sulbactam can bind multiple penicillin-binding proteins (PBPs) found in ABC. They include PBP1a, PBP1b, and PBP3 (47).

## FIGURE 2.

*Xacduro mechanism of action*




Xacduro is a combination of Sulbactam and Durlobactam used to treat pneumonia caused by *Acinetobacter baumannii-calcoaceticus* complex. After intravenous administration, Xacduro enters the infected lung tissue. While Durlobactam is acting as a beta-lactamase inhibitor, Sulbactam can effectively act and penicillin binding proteins (PBPs) to prevent cell wall synthesis. Cell lysis follows resulting in the clearance of the infection.

When these proteins are bound, they are unable to facilitate the synthesis of the bacterial cell wall. Treating ABC with Sulbactam as a β-lactam antibiotic is now ineffective because ABC has developed very efficient hydrolysis of Sulbactam (59). Another mode of resistance for ABC against Sulbactam are in strains that have a rare PBP3 mutation. This mutation was generated in a lab from a clinical isolate and significantly increased resistance to Sulbactam (38). Clinical isolates that contain PBP3 mutations that confer resistance to Sulbactam and Durlobactam remain rare (40). When Sulbactam is used to treat ABC infections in combination with Durlobactam, then Sulbactam regains its ability to interfere with cell wall synthesis (Figure 2).

Durlobactam is a novel diazabicyclooctane class β-lactamase inhibitor. It is considered broad spectrum because it is active against Ambler class A, C, and D serine β-lactamases, all of which are produced by ABC (33), (59). It does not inhibit class B metallo-β-lactamases, which are also found in ABC, but rarely (58), (59). Durlobactam has strong activity against Ambler class D β-lactamases which is not common in other β-lactamase inhibiting drugs (44). The Durlobactam portion of Xacduro is able to inhibit the many β-lactamases found in ABC so the Sulbactam β-lactam antibiotic can directly work towards inhibiting cell wall synthesis of ABC (Figure 2). Selective toxicity is achieved by targeting bacterial β-lactamases and penicillin-binding proteins unique to *Acinetobacter baumannii*, thereby restoring antibacterial activity while sparing mammalian cells.

The FDA prescribing information reported that 36 patients (40%) experienced serious adverse reactions when treated with Xacduro (19). Notably this was lower than the colistin (control) treatment group where 49% of patients had serious adverse reactions. The three most common adverse reactions were liver test abnormalities (19%), diarrhea (17%), and anemia (13%). Like many antibiotics, Xacduro treatment can also lead to the development of *Clostridioides difficile*-associated diarrhea and the prescribing information states to evaluate for this pathogen if diarrhea occurs during or after a Xacduro regimen.

Like Voquezna, Xacduro exemplifies the importance of developing novel drugs that can improve the efficacy of existing treatments. This highlights that there are many types of approaches for drug development and that for drugs to be effective they do not have to work alone. Simultaneously, this demonstrates the difficulty of finding a molecule that can work alone as an effective antibiotic amid the MDR crisis. Since ABC is steadily gaining new resistance traits, it is possible Xacduro will lose effectiveness against ABC in the future. This also highlights the importance of continual development of antibiotics.

## ORLYNVAH AS A TREATMENT FOR UNCOMPLICATED URINARY TRACT INFECTIONS

Orlynvah is a combination of Sulopenem etzadroxil and probenecid that is approved for oral prescription use of uncomplicated female UTIs (42). It was approved by the FDA on February 25, 2024. Sulopenem etzadroxil is an antibiotic component and probenecid is a renal tubular transport inhibitor.

Sulopenem etzadroxil is a novel ester prodrug (68). Ester prodrugs are an inactive version of a drug which can then be activated once they enter the body and are further processed. In the case of Sulopenem etzadroxil, it is hydrolyzed by intestinal esterases to make the active form, Sulopenem. Once active, Sulopenem functions as a β-lactam. The β-lactam ring of the drug alkylates the peptidoglycan-binding protein (PBP) serine residues which inhibits peptidoglycan cross linkage to form the cell wall (Figure 3) (68). This combination demonstrates selective toxicity through β-lactam-mediated inhibition of bacterial cell wall synthesis, a pathway absent in human cells, while probenecid enhances antibiotic exposure without intrinsic antibacterial activity.

Probenecid is a uricosuric agent that was originally developed to decrease the kidney's ability to clear penicillin from the body (51). It was developed in 1949 and approved by the FDA under the brand name Benemid on April 21, 1951 (42), (51). It later was approved and widely used for the treatment of gout because of its ability to decrease uric acid levels in the blood. It became the standard of care for many years until it was determined that decreasing uric acid in the blood did not necessarily improve gout symptoms. Its ability to enhance antibiotic presence in blood was revitalized in the FDA with the approval of Orlynvah. The purpose of dosing Sulopenem and probenecid together is to promote retention of Sulopenem in the blood by limiting renal tubular secretion with probenecid.

Orlynvah is prescribed for women with uncomplicated urinary tract infections caused by *E. coli, K. pneumoniae,* and *P. mirabilis* who do not have other oral antibiotic treatment options (42). It is not approved for the treatment of complicated urinary tract infections. Complicated urinary tract infections are those with a high risk of treatment failure such as those in immunocompromised individuals, men, and UTIs involving the kidneys (53). Complicated UTIs are often caused by bacteria that are unlikely to be susceptible to Sulopenem (42). Since Sulopenem is considered a new drug, the FDA wants to maintain its effectiveness against MDR pathogens, so it is not considered suitable for treating compli-

cated UTIs. Even in uncomplicated UTIs, Orlynvah should only be used to treat infections that are proven to be or likely to be susceptible to Sulopenem.

The FDA prescribing information provides data on the adverse reactions/side effects from taking Orlynvah. Serious adverse reactions only occurred in 6/1932 (0.3%) between two Phase 3 clinical trials (19). There were 21/1932 (1%) patients who discontinued treatment due to adverse reactions. The most commonly reported side effects were diarrhea (10%), nausea (4%), vulvovaginal mycotic infection (2%), headache (2%), vomiting (2%), and abdominal pain (1%). The prescribing information also warns about potential *Clostridioides difficile*-associated diarrhea and exacerbation of gout.

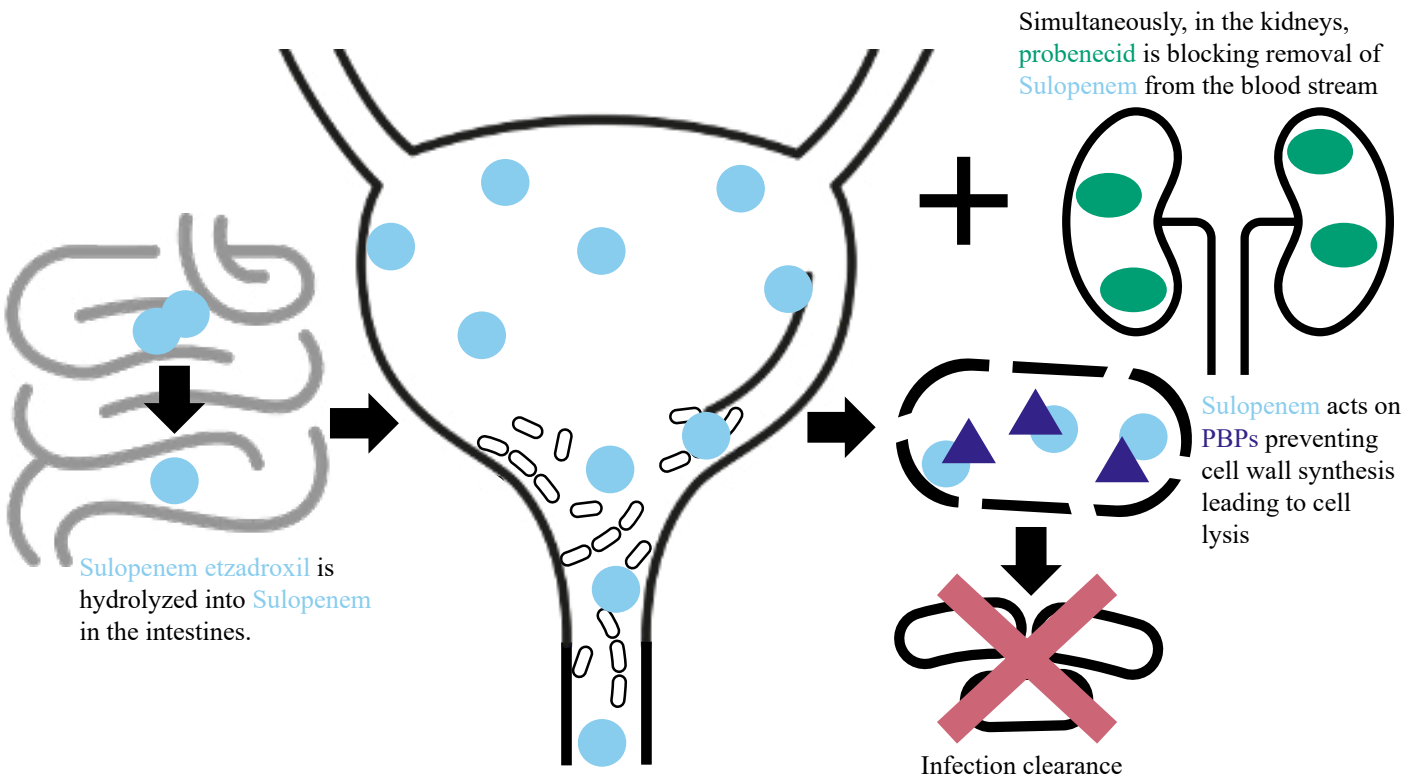
Because of its efficacy against multiple pathogens, Orlynvah is a promising and strong therapeutic option for the treatment of uncomplicated UTIs that could be reapproved at a later date for uses in other types of infections. It is also possible that probenecid will continue to regain relevance in use with other antibiotics.

## ZEVTERA AS A BROAD-SPECTRUM TREATMENT FOR LUNG AND BLOOD INFECTIONS

Zevtera, also known as Ceftobiprole medocaril, is a fifth-generation cephalosporin antibiotic and was developed to combat the increasing threat of antimicrobial resistance (23), (57). It is notable for its broad-spectrum activity against both Gram-positive and Gram-negative bacteria, including challenging pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* (49), (70). On April 3, 2024, the U.S. Food and Drug Administration (FDA) approved Zevtera for the treatment of adults with *Staphylococcus aureus* bloodstream infections (bacteremia),

**FIGURE 3.**

*Orlynvah mechanism of action.*




Orlynvah is a combination of the antibiotic prodrug, Sulopenem etzadroxil and the renal tubular inhibitor probenecid used to treat uncomplicated UTI. Sulopenem etzadroxil and hydrolyzed into Sulopenem in the intestines. Upon circulation to the bladder, Sulopenem acts as a beta-lactam antibiotic, acting on penicillin binding proteins (PBPs) preventing cell wall synthesis and ultimately bacterial lysis. Probenecid simultaneously acts in the kidney as a renal tubular inhibitor to maintain levels of Sulopenem within the body. Image attributions: Bladder icon: Flaticon.com and Intestine icon: Flaticon.com. This figure has been designed using resources from Flaticon.com.

including those with right-sided infective endocarditis; adults with acute bacterial skin and skin structure infections (ABSSSI); and adult and pediatric patients (ages 3 months to <18 years) with community-acquired bacterial pneumonia (CABP) (22). The recommended dosing for this drug is 667 mg of Zevtera by injection. Zevtera has already been approved in several countries for the treatment of hospital-acquired pneumonia (HAP), including MRSA-associated cases, and community-acquired pneumonia (CAP), excluding ventilator-associated pneumonia (VAP) (12).

Ceftobiprole medocaril is a prodrug, meaning it becomes active only after it is converted in the body, specifically in the bloodstream (70). Once activated into Ceftobiprole, it targets penicillin-binding proteins (PBPs), which are essential for building bacterial cell walls (Figure 4). Ceftobiprole exhibits selective toxicity by preferentially binding bacterial penicillin-binding proteins, including resistance-associated PBPs such as PBP2a in MRSA, which have no counterparts in human cells. It binds strongly to PBP2a in methicillin-resistant *Staphylococcus aureus* (MRSA) and PBP2x in *Streptococcus pneumoniae* (23), (70). Besides treating lung infections, Zevtera also shows promise for managing more severe infections, such as bloodstream infections caused by *Staphylococcus aureus* (bacteremia) (26). It has also been used in cases of right-sided infective endocarditis caused by both methicillin-susceptible and methicillin-resistant strains (23). This broader use shows its value in situations where quick and effective treatment is critical.

Ceftobiprole has a wide spectrum of activity. Among Gram-positive bacteria, it works against *Staphylococcus aureus* (both MSSA and MRSA), *Streptococcus pneumoniae*, *Streptococcus pyogenes*, and *Enterococcus faecalis* (23). It also covers Gram-negative bacteria like *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* (23). However, it is less effective against bacteria that produce extended-spectrum β-lactamases (ESBLs) or carbapenem-resistant strains, which may limit its use in those cases (23), (48).

Zevtera is generally well tolerated (64). Common adverse effects include nausea, diarrhea, vomiting, headache, rash, injection site reactions, and elevated liver enzymes (64). Patients with known severe hypersensitivity to Ceftobiprole or other cephalosporins should avoid Zevtera (22), (23). Key safety considerations include the potential for hypersensitivity reactions, central nervous system effects such as seizures, *Clostridioides difficile*-associated diarrhea, and an observed increase in mortality among patients with ventilator-associated bacterial pneumonia (22). The FDA granted Zevtera Priority Review, Fast Track, and Qualified Infectious Disease Product designations for treating *Staphylococcus aureus* bacteremia (SAB), ABSSSI, and CABP (22). These designations highlight its value in addressing urgent medical needs related to severe and drug-resistant bacterial infections.

## EXBLIFEP AS A TREATMENT FOR COMPLICATED URINARY TRACT INFECTIONS

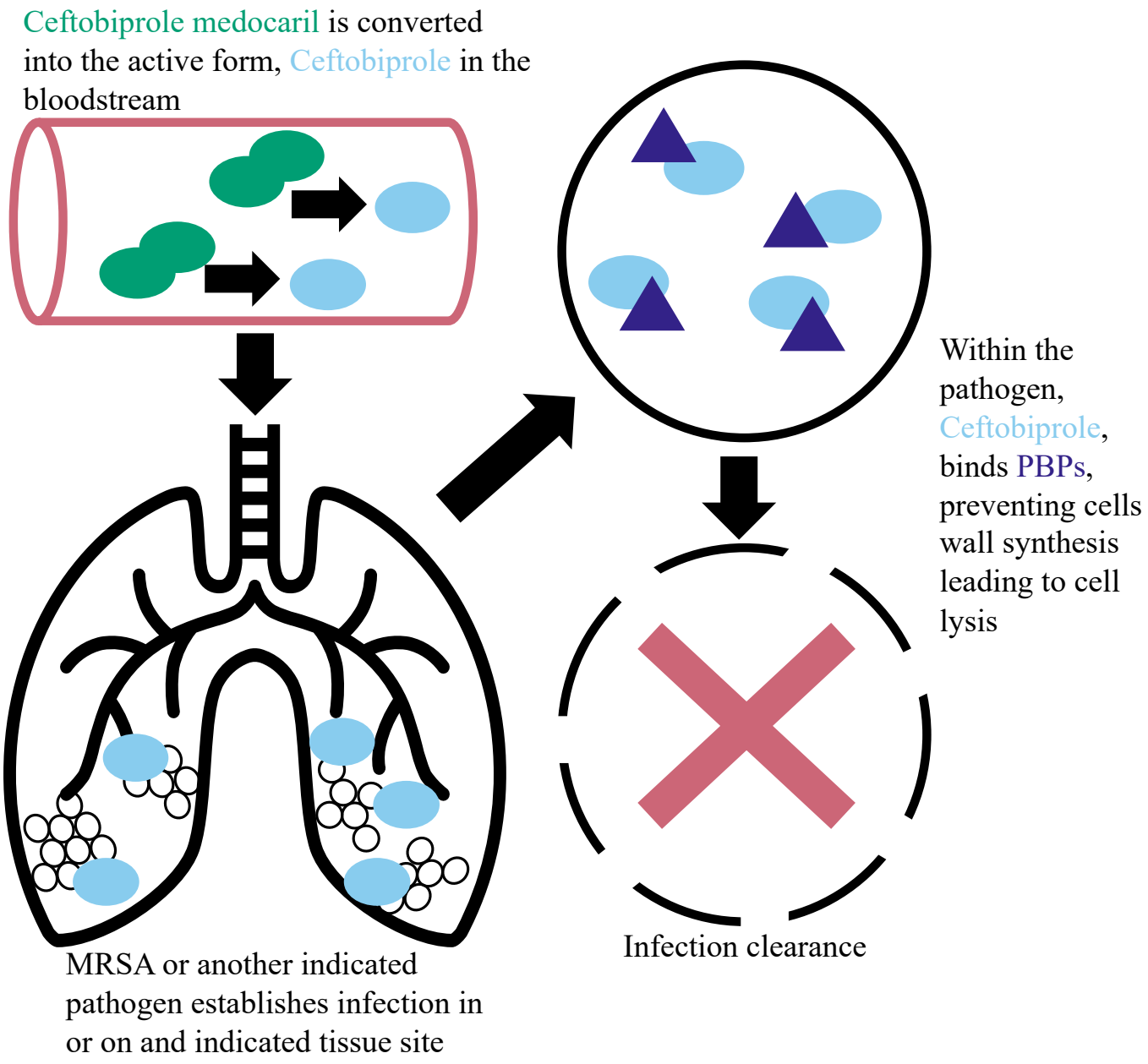
Exblifep (Cefepime/Enmetazobactam) is a novel intravenous antibiotic approved by the FDA on February 22, 2024, for the treatment of complicated urinary tract infections (cUTIs), including pyelonephritis, in adults (17). It is specifically indicated to treat these infections caused by susceptible strains of *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus mirabilis,* and *Enterobacter cloacae* (35). This fixed-dose combination pairs cefepime, a fourth-generation cephalosporin, with Enmetazobactam, a novel β-lactamase inhibitor.

Cefepime interferes with bacterial cell wall synthesis and is effective against many Gram-negative and Gram-positive pathogens (Figure 5) (35). This includes *Pseudomonas*. It binds with PBPs and is stable against some β-lactamases. While cefepime is effective across a broad-spectrum of pathogens, its combination with Enmetazobactam is only approved for Gram-negative infections at this time. Both cefepime and Enmetazobactam are zwitterions, meaning they have a net neutral charge. This improves the ability of cefepime to penetrate the bacterial cell wall. For Enmetazobactam, being a zwitterion increases its potency as a β-lactamases inhibitor compared to other drugs in the same class. As a combination treatment, Enmetazobactam can improve the effectiveness of cefepime against isogenic *E. coli* strains of bacteria expressing class A β-lactamases and shows limited inhibition of select class D β-lactamases, while remaining inactive against class B and C β-lactamases. Selective toxicity is maintained through targeted inhibition of bacterial penicillin-binding proteins and β-lactamases, enhancing activity against Gram-negative pathogens while minimizing effects on host cellular processes.

For the treatment of susceptible pathogens, Exblifep is recommended to be dosed as 2.0 g cefepime and 0.5 g Enmetazobactam intravenously every eight hours (17). The dosing is recommended to be altered for patients with estimated glomerular filtration rates that fall outside of 60-129 mL/min. This regimen should be continued for at least seven days, but it is not recommended to go beyond 14 days. Following the regimen, patients generally tolerated Exblifep well. The most common side effects were increased transaminases (20% of patients), increased bilirubin (7%), headache (5%), infusion site reactions (5%) and diarrhea (4%).

**FIGURE 4.**

*Zevtera mechanism of action.*




Zevtera starts as the prodrug Ceftobiprole medocaril. Within the bloodstream it is converted into the active Ceftobiprole. This antibiotic is indicated for multiple types of infections including lung, skin, and blood infection. Within the infected tissue, Ceftobiprole, a beta-lactam antibiotic is able to bind bacterial penicillin binding proteins (PBPs). This binding prevents cell wall synthesis leading to cell lysis and infection clearance.

In a randomized clinical trial comparing Cefepime and Enmetazobactam to piperacillin and tazobactam, 79.1% of patients receiving Cefepime and Enmetazobactam cleared their infections by day 14 which was higher compared to 58.9% in the piperacillin and tazobactam group, a difference of 20.2% (34). Piperacillin in combination with tazobactam combines an extended-spectrum penicillin, piperacillin, with tazobactam, a β-lactamase inhibitor that protects piperacillin from degradation by β-lactamases. Notably, among patients with infections caused by extended-spectrum β-lactamase (ESBL)–producing pathogens, the clinical cure rate was 73.7% with Cefepime and Enmetazobactam versus 51.5% with piperacillin and tazobactam.

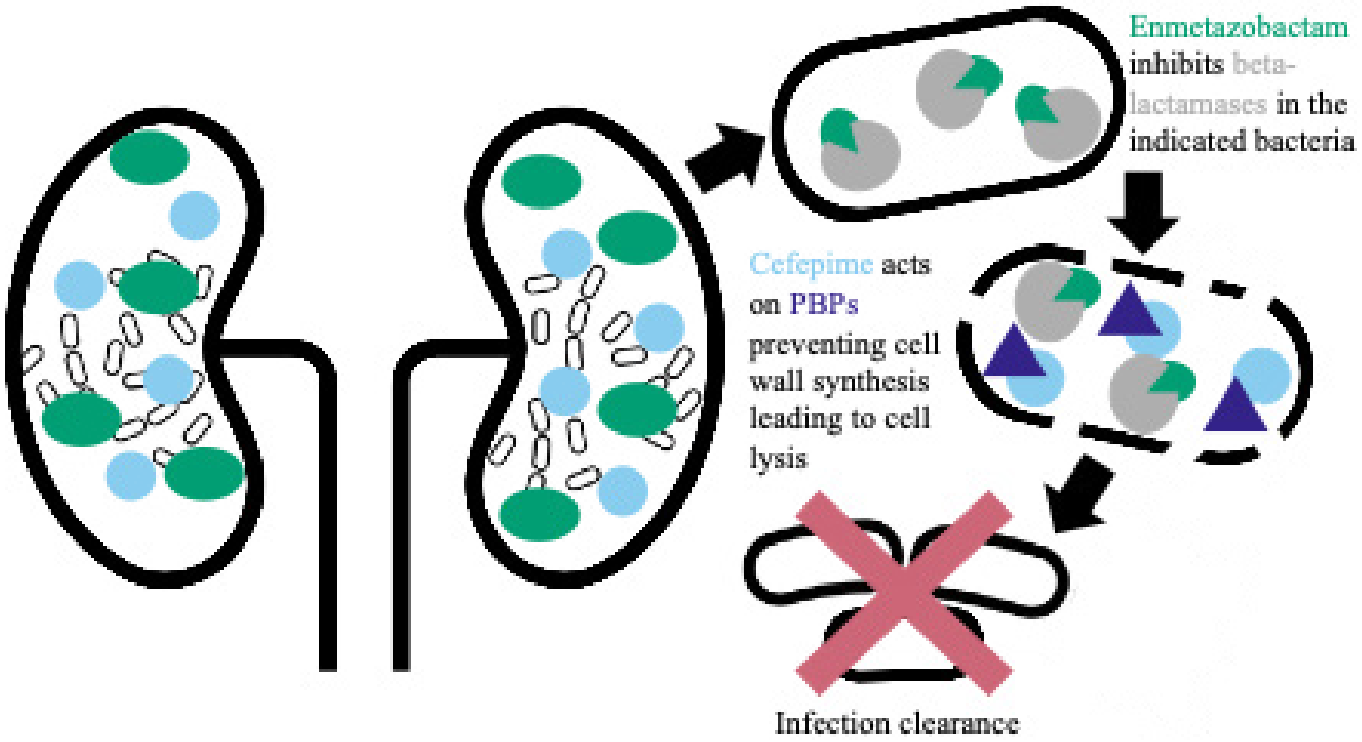
Currently in the EU, Cefepime/Enmetazobactam is approved to treat additional types of infections aside from complicated UTIs. If the United States is to follow in these footsteps, it is possible that Exblifep will be approved for additional uses in the future. It is also possible due to its general broad-spectrum activity Exblifep could be used off-label by clinicians for treatment of infections not involving the urinary tract. This of course would be after careful consideration and input from specialists.

## BLUJEPA AS A TREATMENT FOR UNCOMPLICATED URINARY TRACT INFECTIONS

Blujepa is also known as Gepotidacin. It was approved on March 25, 2025, for the treatment of uncomplicated urinary tract infections (19). This drug is the first in its class of atri-azaacenaphthylenes, a kind of type IIA topoisomerase inhibitor. The inhibition of topoisomerase IIAs prohibits DNA replication leading to bacterial death (Figure 6) (52). Gepotidacin achieves selective toxicity by inhibiting bacterial DNA gyrase and topoisomerase IV, enzymes that are structurally distinct from their human counterparts, thereby limiting host toxicity. Outside of treating uncomplicated UTIs, Gepotidacin is also showing to be non-inferior to a standard ceftriaxone and azithromycin combination treatment for treating

**FIGURE 5.**

*Exblifep mechanism of action*




Exblifep is a combination of the Enmetazobactam, a beta-lactamase inhibitor and Cefepime, a beta-lactam. Exblifep is indicated for the treatment of complicated UTI. Enmetazobactam binds to beta-lactamases in the indicated pathogen allowing for Cefepime to function. Cefepime binds penicillin binding proteins (PBPs) preventing cell wall synthesis. This leads to cell lysis and infection clearance.

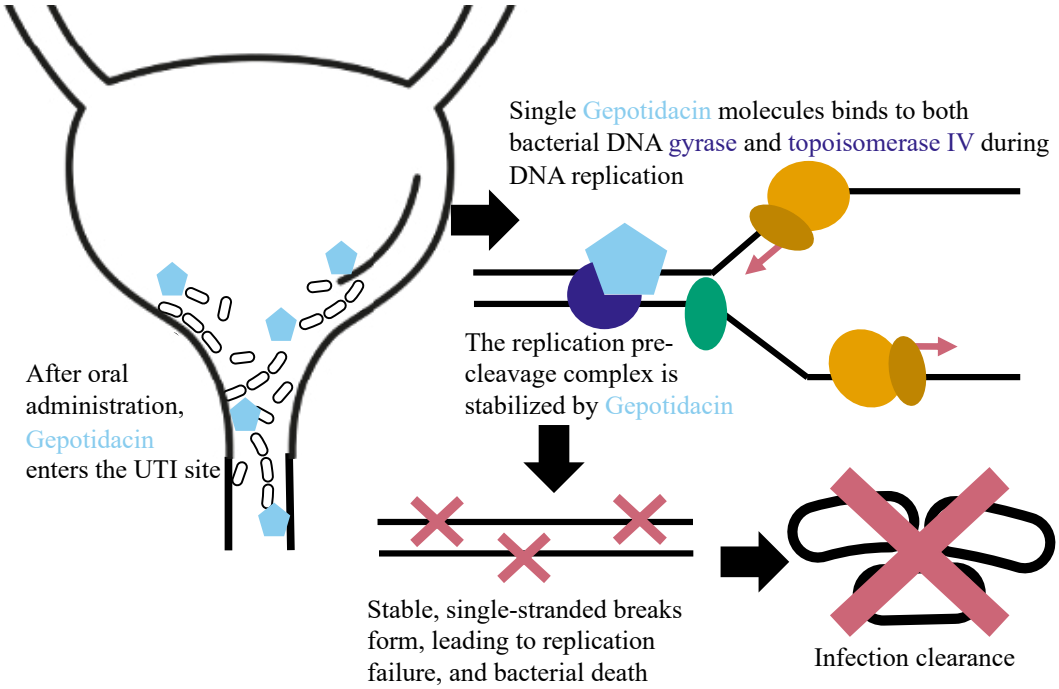
urogenital gonorrhea in a phase III clinical trial. Gepotidacin is administered orally as two 750 mg tablet twice daily as indicated for susceptible *Escherichia coli*, *Klebsiella pneumoniae*, *Citrobacter freundii* complex, *Staphylococcus saprophyticus*, and *Enterococcus faecalis* causing uncomplicated UTIs (19). The treatment is carried out for five days. The FDA recommends taking the regimen after a meal to reduce gastrointestinal side effects.

The FDA states that outside of the aforementioned clinically susceptible bacteria, there are *in vitro* data for other Gepotidacin-susceptible Gram-negative bacteria (19). They include *Citrobacter koseri*, *Klebsiella aerogenes, Klebsiella oxytoca/ Raoultella ornithinolytica*, *Morganella morganii*, *Proteus mirabilis,* and *Providencia rettgeri*. At the time of FDA approval there was no clinical data about the effectiveness of Blujepa against these pathogens.

Blujepa is mostly well tolerated in patients (67). There were no life threatening or fatal side effects during the clinical trials leading up to its approval. The most common side effects were categorized as mild to moderate and were most commonly reported to be diarrhea (14-18%) and nausea (8-11%). Blujepa can cause QTc prolongation, a type of irregular heartbeat, and is therefore not recommended for patients with a history of these conditions or in patients with relevant cardiac diseases (19). Blujepa is also not recommended for patients with liver and kidney impairment. The use of Blujepa can also lead dysarthria, a speech disorder caused from weakness in facial muscles, due to acetylcholinesterase inhibition,

## FIGURE 6.

*Blujepa mechanism of action.*




Blujepa is the brand name for the drug Gepotidacin. Gepotidacin targets both bacterial gyrase and bacterial topoisomerase IV. Gepotidacin stabilizes the pre-cleavage complex that forms during DNA replication. The stabilized complex prevents proper supercoiling and unwinding of DNA leading to single stranded breaks. As a result, replication fails and the pathogen dies, leading to infection clearance. Image attributions: Bladder icon: Flaticon.com. This figure has been designed using resources from Flaticon.com

## TABLE 1.

*FDA-approved antibiotics discussed in this review (2022-2025).*

| Drug(Brand) | Year | Active component(s) | Class/Primary target | Indication |
|---|---|---|---|---|
| Voquezna | 2022 (42) | Vonoprazan*, amoxicillin, clarithromycin (42) | PCAB(acid suppression); β-lactam(cell wall); macrolide (protein synthesis) (55),(2),(46) | *H. pylori* infection (31),(32) |
| Xacduro | 2023 (42) | Sulbactam, Durlobactam* (42) | B-lactam (PBPs); β-lactamase inhibitor (33),(1),(18) | *Acinetobacter baumannii*-calcoaceticus complex infections (33) |
| Orlynvah | 2024 (42) | Sulopenem etzadroxil*, probenecid (42) | B-lactam (cell wall); renal clearance inhibitor (68) | Uncomplicated UTIs (42) |
| Zevtera | 2024 (22) | Ceftobiprole medocaril* (70) | B-lactam (PBPs incl. PBP2a) (23),(70) | ABSSSI, CAP (22),(12) |
| Exblifep | 2024 (17) | Cefepime, Enmetazobactam* (17) | B-lactam (PBPs); β-lactamase inhibitor (35) | Complicated UTIs (35) |
| Blujepa | 2025 (19) | Gepotidacin* (19) | DNA gyrase & topoisomerase IV (52) | Uncomplicated UTIs (19) |

Summary of recently approved antibacterial agents, their active components, primary targets, and clinical indications. Combination therapies are presented as single entries for clarity. *Denotes a newly approved molecular entity.

as well as *Clostridioides difficile* infection, and possible hypersensitivity reactions, including anaphylaxis.

Not only does Gepotidacin inhibit topoisomerase IV, but also gyrase (Figure 6) (19). This new type of drug is an attempt at combating antibacterial resistance to fluoroquinolone antibiotics (6). While both fluoroquinolones and Gepotidacin have the same enzymatic targets, Gepotidacin binds different amino acid residues than fluoroquinolones (25), (43).

Another difference between the drug classes is that fluoroquinolones preferentially target DNA gyrase, whereas Gepotidacin targets gyrase and topoisomerase IV equally (43). By targeting these enzymes, Gepotidacin enhances the stabilization of single stranded DNA breaks over double stranded DNA breaks from supercoiled DNA (28), (43). While fluoroquinolones enhance both types of breakage, double-stranded breaks are more prevalent with this treatment. Overall, Gepotidacin's mechanistic differences are important in overcoming treatment barriers for pathogens that have developed resistance to fluoroquinolones and could be approved for use in treating other infections in the future.

A comparative summary of the recently approved antibiotics discussed above is provided in Table 1.

## FUTURE DIRECTIONS AND PROSPECTS/CONCLUSIONS

The rising threat of antimicrobial resistance (AMR) demands urgent and coordinated global action. While this review has highlighted several novel antibiotics approved by the FDA between 2022 and 2025, each offering distinct mechanisms and improved efficacy against drug-resistant pathogens, the frequency of such approvals remains limited in comparison to the magnitude of the AMR crisis. Despite notable progress, the development of novel antibiotics must become a sustained global priority, backed by policies that encourage innovation and ensure equitable access to these life-saving treatments. Addressing AMR will require not only scientific breakthroughs but also systemic changes that focus on the responsible development, approval, and widespread availability of new antimicrobial therapies.

A key priority is the establishment of sustainable antibiotic development pipelines (39). These pipelines must be designed to overcome economic and scientific hurdles that have historically limited the commercial viability of antibiotics. Incentive structures that reward long-term effectiveness over short-term sales volume, such as delinkage models, should be

more broadly implemented to align public health goals with industry priorities (61).

The role of public-private partnerships and funding initiatives cannot be overstated. Initiatives like CARB-X, GARDP, Tiny Earth, Small World Initiative, and the AMR Action Fund demonstrate the power of collaborative investment in early-stage research and clinical development (4), (13), (30), (60), (65). These partnerships reduce the risks in antibiotic R&D and provide crucial support to small biotech companies and universities, which are often leading innovation.

In the post-antibiotic era, where treatment failures become increasingly common, there is growing interest in alternative antimicrobial strategies. These include phage therapy, antimicrobial peptides, anti-virulence agents, probiotics, and microbiome-based therapies. While still in various stages of development, these alternatives offer the potential to complement or even replace traditional antibiotics, particularly for multidrug-resistant infections.

Another promising frontier is the use of machine learning (ML) and artificial intelligence (AI) in antibiotic discovery. By harnessing large datasets and predictive algorithms, ML can streamline the identification of potential drug targets, optimize the screening of compounds, and even help repurpose existing drugs for new antimicrobial uses (15). Recent breakthroughs in discovering novel antibiotics using AI highlight the potential for these technologies to reinvigorate antibiotic discovery and accelerate the development of new treatments.

Despite these promising directions, there remains a critical need for urgent action at both the scientific and policy levels. Governments, global health agencies, and industry leaders must treat antibiotic resistance with the same level of urgency as other global crises. Comprehensive policy reforms, including stronger surveillance systems, stewardship programs, and streamlined regulatory pathways, are essential to support both innovation and access.

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# Finding Confidence in the Dental School Application Process

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The professional school application process often feels like a test of perfection. Society tells us we need to do everything — every shadowing hour, every leadership role, every volunteer opportunity — to prove we're worthy. It's easy to feel like confidence is reserved for those who seem flawless on paper. But true confidence doesn't come from perfection; it comes from authenticity. Through my own dental school application journey, I've learned that overcoming imposter syndrome and comparison isn't about doing more — it's about trusting that who you already are is enough.

At the start, I felt this constant pressure to be perfect. I worried that if my scores weren't exceptional, if I didn't have a 4.0 GPA, if I wasn't on every leadership board, or if I didn't attend an Ivy League school, I might fall behind. I thought I needed hundreds of shadowing hours, years of dental office experience, and the ability to answer every interview question flawlessly to stand out. The pressure to check every box became exhausting. And while I didn't personally feel less capable than others, I could see how easy it is for people — including myself at times — to get caught up in comparison. During my multiple interviews, surrounded by so many accomplished, driven applicants, it became clear how much that comparison can quietly take away from people's joy and confidence.

Over time, I realized how draining that mindset was. Society loves to make it seem like success belongs only to the people who never stumble — the ones who look perfect, speak perfectly, and perform perfectly. But no one is perfect. Everyone experiences doubt and uncertainty; they just don't always show it. Once I accepted that, I stopped chasing the impossible idea of perfection and started focusing on being authentic. The most confident people aren't those who never fail — they're the ones who keep showing up as themselves, even when it's uncomfortable. And honestly, you can't beat someone who never gives up. Consistency, determination, and faith in your purpose will always outlast comparison or fear.

My perspective started to shift when I stopped trying to sound like the "ideal" applicant and started sharing my real story. In my personal statement, I wrote from the heart about what drew me to dentistry — the balance of science and artistry, and the chance to help people feel confident in their smiles. I talked about what I learned through shadowing, community service, and leadership — not just the achievements, but the challenges that taught me something. When interview days came, I promised myself I wouldn't aim for perfection. I'd just be honest. The more I spoke from experience and shared why this profession mattered to me, the more comfortable and confident I felt. That's when I realized

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authenticity connects far deeper than any polished, memorized answer ever could.

Even though I worked hard for strong grades, solid scores, and meaningful experiences, I came to understand that those things mattered for a bigger reason — not because they made me look impressive, but because they reflected my character. Hard work shows perseverance. Academic success shows curiosity and self-discipline. Leadership shows compassion, initiative, and integrity. Professional schools and employers aren't just looking for perfect résumés — they're looking for people who are kind, dependable, and resilient. Those traits don't come from titles or numbers; they come from who you are when things get tough. Once I understood that, I stopped measuring myself by perfection and started valuing progress instead.

Confidence still took work. I prepared intentionally, reviewed my application carefully, and reminded myself that nerves don't mean weakness — they mean you care. Before interviews, I'd take a deep breath and pray for peace instead of perfection. I also changed how I viewed other candidates. Instead of competition, I saw future colleagues — people chasing the same dream for the same reasons. That shift helped me walk into each interview not as someone trying to prove her worth, but as someone grateful for the chance to share it.

Looking back, the application process taught me more than any test or interview ever could. Confidence isn't built from flawless résumés or impossible standards — it comes from authenticity, self-awareness, and character. Society might pressure us to appear perfect, but true confidence comes from believing that your imperfect, genuine self is enough. Everyone's path is different, and the process works both ways — you're not just finding the right program or opportunity; the right one is finding you. You'll end up exactly where you're meant to be as long as you focus on what you can control: your attitude and your effort. Those two things, more than anything else, define the kind of person you become and the confidence you carry forward.

# Perspective: Teaching through Publishing

**Katie Cooke**

**Ball State University**

In my role as Open Access Publishing Specialist with the Ball State University Libraries, I support the journals published through our open access publishing program. Working with faculty, editors, and authors from all over the world is incredibly rewarding, but I'll admit that my favorite part of the job is getting to work directly with students. Over the past few years, I've especially enjoyed working with Dr. John McKillip and the undergraduate students who produce *Fine Focus* as part of an immersive learning class.

Students gain a unique perspective by producing and publishing an open access scholarly journal. By working directly with faculty in their fields, students learn the proper conventions and practices related to scholarly communications in their field. In addition to supporting our installation of Open Journal Systems, the platform used to produce the journal, I teach students about both open access publishing and considerations for authors from a publishing perspective.

Like all the journals published by the Libraries, *Fine Focus* is an open access journal. This means that all content is freely available immediately upon publication, with no login or payment required by readers. However, articles published through our journals are much more than simple files available on a website.

In my work with students, I walk through some of the signs of credible journals, the basics of open access and scholarly publishing, and the features that they'll use to produce and publish accessible scholarly content.

## OPEN ACCESS PUBLISHING

Open access content is freely available immediately upon publication. Authors publishing online can generally retain copyright to their work, which isn't always the case when publishing with non-open access journals. Students learn about the Creative Commons license that all *Fine Focus* content is published with, which allows authors to retain the copyright to their work while sharing it with others. Students also learn about identifiers such as ORCID, and how these are increasingly important to researchers and scholars.

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### DISCOVERY AND CITATION CHAINS

When articles are published, they are indexed in several key scholarly discovery platforms. This ensures that *Fine Focus* articles will be included in the search results of other scholars. Titles, abstracts, and keywords are indexed, meaning that it's important to make sure those fields are well written to ensure the discoverability of each article. Good titles can be hard to write, and a well written one will give a good overview of the article contents. Abstracts both give the reader an idea about what's included in the article, but they are also an important part of discovery process. Many library search tools offer brief descriptions of articles; these are almost always pulled directly from the abstract.

One strategy to select powerful keywords (sometimes called subjects) is to start with a research database related to the article subject. *Fine Focus* authors might start with Biological & Agricultural Index Plus, for example. Students unsure of the best databases in their field are encouraged to reach out to librarians, who can guide students to excellent resources.

Many databases feature lists of the keywords they use; this is a great place to find effective keywords for an article. Sometimes these lists are called database thesauri or subjects authority. Instead of guessing what good keywords would be, researchers can see what publishers in the fields use as keywords. For example, a researcher writing about bacteriophage could check the Biological & Agricultural Index Plus Subjects Authority and find that there are many terms for that concept; choosing a specific term from the list will both better describe the article and help other researchers find it.

The concept of the citation chain is new to many students, and it reinforces the importance of properly crediting the work and ideas of others. A good practice is to display the bibliography/references used in an article on the article landing page. This allows readers to quickly see the articles the author consulted.

Additionally, those references will be registered with the article metadata. By doing so, the article will be part of a "citation chain" - commonly seen in discovery tools as "Cites/Cited by". These tools allow researchers to easily discover the works cited in an article, as well as where that article is itself cited. Properly citing the work of others is vital to the scholarly publishing process, and by registering references, authors follow the best example.

### JOURNAL PUBLICATION

*Fine Focus* is produced using the Open Journal Systems platform, an open source system used by publishers worldwide. This platform supports the entire publication process, from article submission, through the peer review process, and finally through production and publication.

Students learn about the submission process, and how articles need to be submitted without identifying information. When an article is accepted for review, it begins the peer review process. *Fine Focus* uses a double blind peer review process, which means reviewers don't know the identity of the author, and the author doesn't know the identity of the reviewers. Students learn about the role and the importance of the reviewer, and they see how the system reinforces the anonymity of the process.

Discussions between reviewers and editors take place in the system, and authors can sometimes submit multiple revisions, each of which can be subjected to another round of peer review. When the article is ready for publication, it is sent to the designer for page layout.

At this stage, students learn about the importance of proofreading, and what they should pay special attention to. Many people are surprised to learn that formatting can easily be changed along the manuscript process, especially when auto-save features might save accidental mistakes. Italics, superscripts, subscripts, and special characters are especially important to double check in scientific writing.

## CONSIDERATIONS FOR AUTHORS

As part of my role, I assist several journals with page design services. Over the years, I've noticed many common problems that cause problems when manuscripts are prepared for publication. Students learn about several of these, and how they can lead to errors or inaccessible content.

### GRAPHICS

When submitting a manuscript, it's perfectly fine to paste an image into the manuscript for the review process. When the article is prepared for publication, though, authors should always send the highest quality version of their images in standard image formats (jpg, png, svg, etc). Images almost always need to be resized, and may have lost quality if they've been copied and pasted several times, and sending quality image files allows the designer to resize as needed without losing quality.

Charts, graphs, and any image containing text should be prepared as vector files if possible. A graph saved as a vector file will resize without getting blurry, and more importantly, so will any text included in the label. Students learn about considerations related to accessibility; colors used in charts

should account for colorblindness and people printing in grayscale, for example.

### TABLES

Tables need to be formatted as tables, with table headings, using the tools included in standard word processing applications. Authors using manual tabs and hard returns to mimic the look of the table might not realize those breaks will skew in the design process, and will not be read correctly in text readers. If designers don't notice the problem and have to manually fix it, errors can easily be made at this point. Additionally, improperly formatted tables won't pass accessibility standards.

### STYLES

Using styles in word processing or desktop publishing programs ensures consistency, increases accessibility, reduces the chances for error, and saves time. The text in this paragraph uses a style called "body text", while the "Styles" subheading uses the style "heading3". Styles ensure all articles use the same consistent formatting, and they can also be used to generate the tags needed in accessible PDFs.

Styles remove the need to use hard returns and characters for formatting. The most common error I have seen authors make relates to references, which generally use hanging indents. Just as authors shouldn't use manual line breaks and tabs to mimic the look of a table, they shouldn't use them to produce a properly formatted reference list. Instead, authors can create a "references" style, using hanging indents. This ensures that references will appear correctly during the design process, and even more importantly, they will be registered correctly at publication. Figure 1 illustrates a reference using manual line breaks, and Figure 2 shows how those manual line breaks translate into easily-missed incorrect references.

## CONCLUSION

Classes like Fine Focus are truly immersive experiences, and I find it incredibly rewarding to support students as they produce the journal. They've learned in classes about the importance of bibliographies, crediting others, and following style guides, and they gain a completely new perspective when they serve as journal producers and publishers. Working with students in this role also helps me support the other journals and platforms I work with, and I'm looking forward to seeing what lies ahead for the journal.

**Figure 1.**

*References created in a word processing program*

Miller, G., Necessary, K., Burchell, R., Iwase, Y., Lautensack, N., Russell, B., … Sattley, M. (2024). Positive effects of Moringa oleifera and Moringa stenopetala seed and leaf extracts against selected bacteria. *Fine Focus, 10*(1), 58–73. https://doi.org/10.33043/FF.10.1.58-73

Swain, A., Colburn, E., De Andrade, K., Rosales, P., Addorisio, S., Stuart, T., … Pina, C. (2025). Analyzing selected FDA approved drugs for effects on template switch mutagenesis in E. coli. *Fine Focus, 11*(1), 12–23. https://doi.org/10.33043/rq7a84ax

Note. Created in Microsoft Word, these references look identically formatted, with a left justified first line and indented second and third lines.

**Figure 2.**

*References imported into a desktop publishing program, with hidden formatting characters displaying*

Miller, G., Necessary, K., Burchell, R., Iwase, Y., Lautensack, N., Russell, B., … Sattley, M. (2024). Positive effects ¶
of Moringa ¶
oleifera and Moringa stenopetala seed and leaf extracts against selected bacteria. Fine Focus, 10(1), 58–73. ¶
https://doi.org/10.33043/FF.10.1.58-73¶
Swain, A., Colburn, E., De Andrade, K., Rosales, P., Addorisio, S., Stuart, T., … Pina, C. (2025). Analyzing ¶
selected FDA approved drugs for effects on template switch mutagenesis in E. coli. Fine Focus, 11(1),

Note. The references shown in Figure 1 have been imported into a desktop publishing program. Because the author used hard returns and manual tabs for the first reference, that reference is now four incomplete lines instead of one correctly formatted reference. The second reference used a reference_list style featuring hanging indents and is correct.

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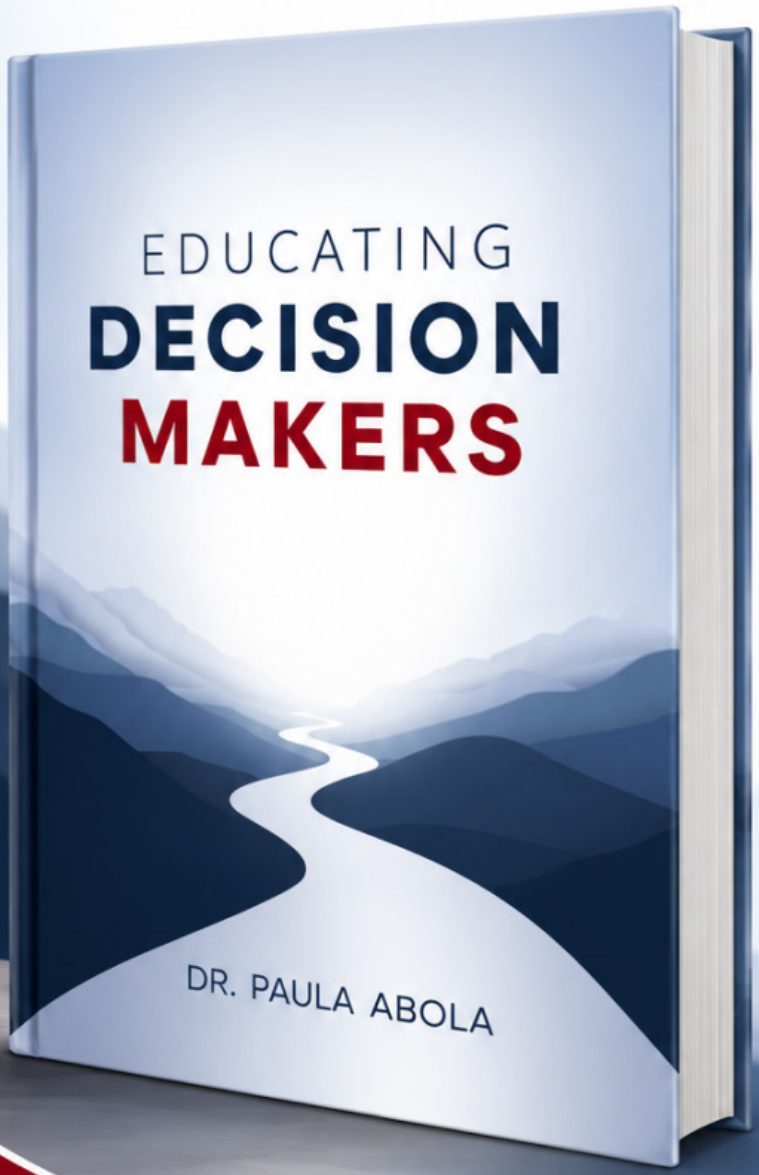


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