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Antibiotic resistant bacteria in natural environments within Kentucky.

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Antibiotic Resistant Bacteria in Natural Environments within Kentucky

By

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Abstract

Rates of antibiotic resistance in bacteria are rapidly rising; this is, in part, due to overuse of antibiotics resulting in a great burden on the U.S. healthcare system. With the rise of resistant bacteria, a large-scale outbreak of antibiotic resistant bacterial infections may also occur. Research on developing antibiotics has also decreased. Thus healthcare is at a great disadvantage in the arms race against bacteria. The environmental reservoirs of antibiotic resistance are currently being explored in Kentucky, in which bacteria have been collected from a broad spectrum of natural environments to analyze their antibiotic resistant capabilities and their interactions with other environmental organisms. Twenty-seven different genera are represented among 138 antibiotic resistant bacterial isolates collected and ten of the genera are known human pathogens. Experiments on representative isolates were conducted to examine the (i) antibiotic resistance profiles, (ii) ability to consume antibiotics as sole carbon sources, (iii) interaction with two plant models, and (iv) interaction with two fungal species. Results obtained from these experiments showed that a representative bacterial panel of strains had high sensitivities to two of the nine antibiotics tested (rifampicin and tetracycline), and low resistance to one of the antibiotics tested (colistin). However, most of the isolates in our panel were unable to consume antibiotics as a carbon source suggesting that they tolerated the antibiotics rather than metabolized them. Additionally, the interaction between our strain panel and its putative ecological partners showed low levels of pathogenicity in plant models but a differential competitive ability against two fungal strains. These results indicate that our small bacterial representative panel can be used for future studies as they may represent larger populations of native bacteria found in Kentucky.

Introduction

Antibiotic resistant bacteria are quickly emerging worldwide due to overuse or misuse of antibiotics. The Centers for Disease Control and Prevention (CDC) has classified a number of bacteria that are becoming resistant to many available antibiotics and potential threats to the U.S. healthcare system, patients, and their families [40]. The quick increase in resistant bacteria is due to the rapid spread of resistance between bacteria over time.

Mechanisms for Antibiotic Resistance Spread

The emergence of antibiotic resistant bacteria is often due to bacteria obtaining plasmids (or other mobile DNA elements) containing genes whose protein products confer antibiotic resistance. A plasmid is a small, circular, double stranded DNA molecule that is separate from the larger chromosomal DNA within a bacterium. Due to the distinct separation of the chromosomal DNA and plasmid, plasmids are considered to be non-essential for basic cell function but instead offer advantages to survival. For example, plasmids bearing antibiotic resistance cassettes, which are mobile genetic elements that contain genes and recombination sites allowing for genes to be removed or inserted into the cassette and the plasmid itself, may confer resistance to a bacterium in the presence of a specific selective agent. Not only do plasmids sometimes contain antibiotic resistance genes, but they may also contain genes that encode a structure known as a conjugative pilus, which would allow a bacterium to transfer plasmids with another bacterium through a process known as conjugation. Conjugation allows a donor bacterium to distribute plasmids (potentially containing antibiotic resistance cassettes) to neighboring bacteria, therefore making them newly resistant to certain antibiotics [4].

Conjugation and other methods for DNA transfer between bacteria [i.e. transformation (uptake of free DNA) or transduction (DNA transferred from bacteriophages)] could result in the emergence of opportunistic, antibiotic resistant bacterial pathogens that could now cause morbidities in humans where initially the bacterial strains were harmless. Not only can it create resistant bacteria but also multi-drug resistant bacteria otherwise known as "super bugs" such as methicillin resistant *Staphylococcus aureus* (MRSA) or extremely drug resistant (XDR) *Klebsiella pneumoniae* [3]. Some strains have even been reported to be pan resistant – resistant to all antibiotics tested [24]. Since many of the opportunistic, antibiotic resistant bacteria causing human infections are genetically distinct rather than clonal, as one would expect from an outbreak spread patient-to-patient, it is highly likely that many of the resistant bacteria are acquired from environmental sources [25].

Pathogenic Gram-negative bacteria are deemed difficult to eradicate due to the unique composition of their cell wall, having an outer and inner membrane, and porins on the outer membrane [36]. The outer membrane allows resistance to many antibiotics that are effective against Gram-positive bacteria. Many Gram-negative bacterial strains are becoming increasingly resistant to many or most available antibiotics; thus placing a great burden on the U.S. healthcare system [40]. Research leading to the development of new antibiotics has also been reduced [10; 29; 33] because of the large cost of research, development, and clinical trials.

Cystic Fibrosis and Antibiotic Resistant Pathogens

Cystic Fibrosis (CF) is a genetic disorder that results in persistent lung infections and leads to gradually decreased capability to breathe in the patient. It is caused by a defect in the human *CFTR* gene, which encodes a chloride ion transporter. The result of any one of a number

of potential mutations in this gene is the build-up of viscous mucus on the linings of the lungs and gastrointestinal tract. This sticky mucus is very carbon rich and thus a good food source for bacteria. Patients with CF experience bouts of bacterial pneumonia over the course of their life leading to lung scarring and pulmonary decline. Many people with CF are treated prophylactically and during exacerbation events with high doses of antibiotics [32]. Two particular antibiotics that have been commonly used to treat Gram-negative bacterial infections, specifically in CF patients, are tobramycin and colistin [5; 11; 13; 20; 22; 28; 32; 34]. Tobramycin, an aminoglycoside antibiotic that disrupts protein synthesis in bacteria, has been effective at treating Gram-negative bacterial infections [7] and is commonly used to treat CF patients prophylactically through nebulization. Additionally colistin, a polymixin antibiotic that interferes with cell wall synthesis in bacteria, has been reassessed as being an option for treating multi-drug resistant bacterial infections [21] and is a common antibiotic given to CF patients during periods of lung decline due to pneumonia. However, some CF pathogens have the ability to resist tobramycin and/or colistin treatment [5; 23].

Resistance to tobramycin can be mediated by two main mechanisms. First, bacteria can alter the permeability of their cell membrane thus leading to a decrease in the uptake of the antibiotic [23; 29]. Mechanisms for impermeability are still undetermined though two proposed mechanisms are alterations in the lipopolysaccharide or changes in outer membrane proteins [23]. Second, tobramycin resistance can evolve through enzymatic modifications of the antibiotic using *N*-acetyltransferases (which catalyze acetyl CoA-dependent acetylations of the antibiotic) and *O*-adenyltransferases (which catalyze ATP-dependent adenylation of tobramycin) – both of which cause a structural modification that prevents the antibiotic from binding to the ribosome [29]. Alteration of the permeability in the membrane is the predominant resistant mechanism

against tobramycin with a small percentage of the resistant bacterial population having enzyme modification as the resistant mechanism. An even smaller population utilizes both mechanisms for resistance [23].

Resistance to colistin can emerge in several different ways, two of which are similar to tobramycin resistance. One method in resistance is the bacterium's ability to reduce the uptake of the antibiotic by releasing the antibiotic back into the environment quickly with the aid of efflux pumps. Colistin resistance can also be mediated through enzymatic modification of the antibiotic which reduces/prevents it from binding to the target, cellular phospholipids [21]. One unique resistance to colistin not seen in tobramycin resistance is the mutation the *ipxA*. This gene is essential for biosynthesis of lipid A, which is the precursor for the biosynthesis of lipopolysaccharide that serves as a major component of the outer cell membrane of the Gram-negative bacterium. The mutation leads to absence of production of lipid A and renders colistin ineffective since colistin acts by binding to lipid A in order to function [26]. Though this mutation has only been reported in a few bacteria such as *Acinetobacter baumannii* [26].

Methods to Studying Antibiotic Resistance

One common method in assessing a bacterium's susceptibility to an antibiotic is the minimum inhibitory concentration (MIC) assay. An MIC assay is performed by subjecting a bacterial population to decreasing concentrations of an antibiotic. This is normally done in 96-well plates that are incubated for 24-48 hours. After the incubation period, the lowest concentration of antibiotic that causes no visible growth of the bacterium in a well is the MIC of the bacterium [15]. MIC is a technique that provides insights into the extent of resistance towards an antibiotic of a bacterial isolate though it is limited because it is based on observable growth.

For most bacteria, concentrations of up to 100,000 bacterial cells per milliliter are not observable with the naked eye; thus, there may be limited but unobservable growth in a well in response to an antibiotic.

Despite this limitation, the results obtained from an MIC assay can be further interpreted as to how sensitive or susceptible the bacterium is against a particular antibiotic. Once a bacterium has been confirmed as resistant towards a particular antibiotic, the bacterium can be further studied in search of the molecular mechanism that conveys resistance. A common and useful method in closely studying mechanistic steps in resistance would be the use of polymerase chain reaction to identify known antibiotic resistant cassette markers. One limitation of this method, however, is that it will not identify unknown or novel mechanisms.

Other Means to Studying Antibiotic Resistant Environmental Isolates

What are the ecological roles of these antibiotic resistant bacteria? There are numerous ways to answer this question. This study will focus on the interaction of these isolates with fungi and plants that may occupy the same environmental niche as the bacteria.

Bacteria interact with plants in a variety of ways. Plants and bacteria can form beneficial symbioses such as those rhizobial bacteria that can produce nitrogen-fixing root nodules on the root hairs of leguminous plants. Conversely, the relationship can be adversarial as in the case of plant pathogens. In this case, the bacterium must penetrate the thick plant cell walls in order to cause destruction of tissue and dissemination though the plant. Researchers commonly study plant models such as *Arabidopsis* or cultivated vegetables to study plant pathogenesis [37]. The latter plant models include using onions or lettuce leaves to simulate live plants in a research laboratory.

Mechanistic studies have shown that some pathogenic bacteria produce similar subsets of virulence determinants that cause disease in animals and plants [30]. In many cases, plants have evolved defense mechanisms such as the use of phytochemicals to combat pathogenic bacteria that are different from animal defenses. From this, another beneficial use of plant models would be the discovery of a possible source of new therapeutics (i.e. phytochemicals) [30].

Another method to analyze these bacterial isolates would be to study microbial interactions because many of the antimicrobials commonly used in clinics are actually natural products derived from environmental organisms thought by some to be involved in interspecies communication. For example, the genus *Streptomyces* accounts for 80% of all antibiotics approved for human use and includes streptomycin, cephalosporins, chloramphenicol, neomycin, tetracycline, nystatin, cycloserine, vancomycin, kanamycin, fosfomycin, and the newer antibiotics daptomycin and platensimycin [31]. Additionally, fungal species can produce antibiotic (*e.g.* penicillin – described more below). Microbial interaction between microbes can be mutualistic, commensal, competitive, or inhibitory depending on the microbes involved.

In addition to antibiotics produced by fungi, some of the antibiotics that have been used clinically since the 1920's were developed from fungi. Penicillin was discovered and developed from a study of bacterial interactions with the *Penicillium* genera of fungi [16]. *Penicillium* species evolved mechanisms to promote survivability, increase competitiveness, or to act as a defense mechanism against other microbes [28]. One mechanism was the secretion of antibacterial compounds, which later evolved into penicillin. There are many fungi that reside in natural environments alongside with bacteria and many of the microbial interactions are competitive or inhibitory. One pathogenic fungus used in this study, *Aspergillus fumigatus*, has been shown to produce potent antibacterial compounds [12; 38; 41] and is readily found in the

soil or decaying organic matter. On the other hand, the human commensal fungus *Candida albicans* has been shown to work in concert with the human microflora to build biofilms, particularly in the oral cavity [6] but has also been isolated from soils, waters, and in association with plants [27]. This suggests that fungi and microbes can maintain mutualistic relationships as well as competitive/inhibitory relationships; however whether they are actively growing in such environments or are just deposited from animal feces is still unclear.

Another method to analyze these bacterial isolates would be to study the mechanism that conveys resistance. Resistance mechanisms are normally on the molecular level involving changes to certain structures or involving enzymes. Recently, there has been an increase interest in bacteria that are able to not only tolerate the antibiotic but also metabolically consume it. It has been observed that certain clades of bacteria can utilize antibiotics as carbon sources; that is, these bacteria have the ability to consume the antibiotics as food [9]. Growth of a bacterium on the medium containing the antibiotic as a sole carbon source indicates that the bacterium is resistant to the antibiotic and may be either able to breakdown the antibiotic for metabolic consumption or fix CO_2 as an autotroph. Fully understanding how the bacterium renders an antibiotic useless or less effective allows researchers to pinpoint and target essential molecular steps in hopes of eradicating or controlling the bacterium.

With the rise of antibiotic resistant bacteria, the push for antibiotic research or treatment against such bacteria must be maintained to prevent continuing instances of pan-resistant organisms and outbreaks. The goals of this study are to investigate the antibiotic resistant capabilities of the bacterial isolates collected in Kentucky and examine the ecological roles of these organisms in their environment. *The main hypotheses of this project are that the bacterial isolates obtained in natural environments within Kentucky are resistant to many antibiotics and*

that bacterial isolates found in soil environments will be able to consume antibiotics. We further

hypothesize that some bacterial isolates will be plant pathogens and some bacterial isolates will

be inhibited by antibacterial compounds produced by the fungi.

Materials and Methods

Sampling Antibiotic Resistant Bacterial Isolates

Making LB Agar Plates Containing Antibiotics

In order to study the bacteria of interest, Lennox LB (Luria-Bertani) agar plates were

prepared. Plain Lennox LB agar is a nutrient medium essential for microbial growth and was

made by dissolving 35 g of Lennox LB agar powder (IBI Scientific) into 1 liter of ultrapure

water. Lennox LB agar contains 15 g of agar, 10 g of tryptone, 5 g of yeast extract, and 5 g of

sodium chloride per liter. The medium was then autoclaved and placed in a 55°C water bath until

cooled. To select for only antibiotic resistant bacteria, tobramycin and colistin were added into

the medium (to final concentrations of 25 mM and 9 mM for colistin and tobramycin

respectively) with swirling to help distribute them evenly. The medium was then poured out into

plates and the plates were flipped after solidifying for overnight drying. Flipping the plates

prevents possible contamination from water droplets that condenses onto the lid due to the heat

from the medium.

Sampling and Collecting Bacterial Isolates

A group of undergraduate and post-bac students and I surveyed natural environments

within Kentucky that had a low human activity. The group was divided into four teams, each

with a specific natural environment to survey for bacteria (*i.e.* association with animals or fungi,

plants, water, and soils). The teams spread out and traveled to different areas within Kentucky stretching east from Louisville to Ashland and south to Elizabethtown (**Supplemental Figure 1**). Being part of the team to survey plants, our methods in sampling and collecting bacterial isolates were slightly different than the other three teams. Plants were surveyed in multiple different areas in Louisville parks (**Supplemental Figure 2)**. Boston lettuce, broccoli, and an ear of a corn were obtained from groceries procured at a Kroger grocery store (4303 Winston Ave, Convington, KY 41015). The plants chosen were sampled by swabbing the external surfaces with a sterile cotton swab damped with sterile ultrapure water. The swab was then used to inoculate an LB plate supplemented with colistin and tobramycin antibiotics and incubated at 37°C in order to grow cultures of the bacteria for further study within the lab.

Determining Resistance to Additional Antibiotics by Plating

The bacterial isolates that grew on colistin- and tobramycin-containing LB agar plates were transferred to LB agar plates containing up to three additional antibiotics (*i.e.* carbenicillin, erthryomycin, and irgasan). Plates were made similarly to as described above but with different combination of antibiotics. Plates contained a combination of carbenicillin (500 μg/mL) and irgasan (25 μg/mL); erthryomycin (500 μg/mL) and irgasan (25 μg/mL); or all five antibiotic used thus far. Resistance to these antibiotics was determined by examining the streaked area for visible growth of bacteria. Individual colonies (in contrast to full streaks) were not used as an indication of antibiotic resistance because, individual colonies are more likely the result of mutations in the face of selection than full streaks, which indicates that antibiotic resistance is common among the colonies.

Identification of Resistant Bacterial Isolates

Extraction of Genomic DNA from Bacterial Isolates

   Half of the identification was done by a group of undergraduate and graduate students in a summer research lab experience (including myself). The remaining half was done by a post-bac student, Eric Curtsinger, and myself. Genomic DNA (gDNA) was extracted from each bacterial isolate using a DNeasy Blood and Tissue kit (Qiagen). The concentration of each gDNA sample was determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific) at 260 nm. Concentrations were adjusted to 50-600 ng/mL to ensure that there was enough gDNA for sequence amplification.

Polymerase Chain Reaction of the Small Ribosomal rRNA Gene

   The 16S ribosomal rRNA gene was the target for amplification with each isolate gDNA sample. This gene is commonly used to identify bacterial strains [17]. Two different high-fidelity polymerases were used to amplify the 16S gene region for each bacterial isolate gDNA sample to increase the accuracy of sequencing. However each halves of the bacterial isolates had a different polymerase. For each gDNA, a master mix was made using the HotStar polymerase (Qiagen). Each reaction (or isolate reaction) contained 10 μL of Q-solution, 10 μL of 5x solution, 2 μL of upstream 16S universal primer 27F (5'- AGAGTTTGATCMTGGCTCAG -3′; [17]; 10 pmol/μL), 2 μL of 16S universal downstream primer 1392R (5′- ACGGGCGGTGTGTRC -3′; [17]; 10 pmol/μL), 1 μL of HotStar polymerase (2.5 units/μL), 25 μL of sterile ultrapure water, and 1 μL of the template (bacterial gDNA). A second master mix consisted of 10 μL of 5X KAPA solution (KAPA Biosystems), 2 μL of upstream primer 27F, 2 μL of downstream primer 1392R, 1 μL of KAPA polymerase (1 unit/μL), 1.5 μL of dNTP mix

(10 mM each) , 33.5 μL of sterile ultrapure water, and 1 μL of template. The reactions were then subjected to PCR using different programs for the two polymerases based on manufacturer's recommendations. The program for the PCR reactions with HotStar was: 5 min at 95°C; 35 cycles of 1 min at 94°C, 1 min at 55°C, 2 min at 72°C; 10 min at 72°C; and holding at 4°C. The program for reactions using KAPA was: 5 min at 95°C; 30 cycles of 1 min at 98°C, 1 min at 60°C, 2 min at 72°C; 10 min at 72°C; and holding at 4°C. After the PCR programs were completed, the products were electrophoresed on a 1% agarose gel, and each sample was examined for the presence of a ~1400 bp band corresponding to the 16S rRNA gene. Samples containing only one strong band of the proper size were purified using the QIAQuick PCR purification kit (Qiagen) in order to remove any traces of remaining primers, enzymes, and salts prior to sequencing.

Sequencing and Identification

The purified PCR products were sequenced at Macrogen (Baltimore, MD) using the Sanger method to determine the 16S DNA sequences. These DNA sequences were compared to DNA sequences in two databases, GenBank (a national gene and protein database) using the BLAST (Basic Logic Alignment Search Tool) algorithm [1] and the RDP (Ribosomal Database Project, only contains 16S gene sequences) [8; 42]. By comparing experimentally determined 16S rDNA sequences from the isolates with sequences from databases, the most closely related genus/species for all isolates was established. In all cases, the most closely related species showed at least a 98% identity to the query sequence suggesting a close match.

Selection and Preparation of Bacterial Isolates

Of the 138 bacterial isolates, 36 isolates were chosen to represent the 27 genera to be tested in the MIC assays, lettuce infection assays, fungal-bacterial interaction assays, and antibiotic single carbon source assays. The isolates were selected by choosing 1-3 representative isolates from each of the 27 genera. Genera containing more than five isolates had at least 2-3 representative isolates selected. The classification of the 36 isolates has been abbreviated for simplicity (**Supplemental Table 1**). Bacterial isolates were prepared by obtaining the isolates from frozen 20% glycerol stocks and streaking them on LB agar plates. The plates were then incubated at 37°C for 24-48 hours. After incubation plates wrapped with parafilm and stored at room temperature for subsequent experiments.

Minimum Inhibitory Concentration (MIC) Assay

Preparation of Antibiotic Solutions

Antibiotic solutions were prepared for use in 96-well plates for the minimum inhibitory concentration assay. Each antibiotic stock solution concentration was 50 mg/mL. Each antibiotic was prepared by dissolving 50 mg of the antibiotic into 1 mL of an appropriate solvent (**Table 1**). Antibiotics that were dissolved in water were then filtered through a 22 μm syringe filter to ensure sterility. Antibiotic solutions were then aliquoted and stored at -20°C. Tubes containing light-sensitive antibiotics were wrapped with aluminum foil to prevent any light-induced degradation.

Table 1. Antibiotic Information.

Antibiotic	Classification	Mode of Action	Target Type of Bacteria	Solvent - Solubility (mg/mL)
Chloram-phenicol	Others	Inhibits translation by blocking peptidyl transferase on the 50S	G+ and G-	Ethanol – 50
Colistin	Polypeptides	Disrupts the outer cell membrane	G-	Water – 50
Kanamycin	Aminoglycosides	Binds to 70S ribosomal subunit and inhibits growth of the bacterium	G-, some G+	Water – 50
Nalidixic Acid	Quinolones/ Fluoroquinolone	Interferes with RNA and protein synthesis	G+ and G-	Chloroform – 50
Penicillin	Penicillin	Inhibits peptidoglycan synthesis in cell walls	G+ and G-	Water – 100
Rifampin	Others	Inhibits RNA polymerase	G+ and G-	Chloroform – 349
Tetracycline	Tetracyclines	Inhibits protein synthesis and ribosomal binding of aminoacyl-tRNA	G+ and G-	Water – 50
Tobramycin	Aminoglycosides	Inhibits formation of the 70S ribosomal complex	G-, some G+	Water – 50
Trimethoprim	Sulfonamides	Interferes with DNA synthesis	G+ and G-	DMSO - 50

Normalizing Bacterial Isolates

Since the bacterial isolates used in this study are different in terms of growth rates or cell

size, normalizing the bacterial isolates based on culture density allows the MIC data to be

compared to one another. Each bacterial isolate was inoculated into a test tube containing 5 mL

LB liquid and incubated at 35-37°C on a roller drum for approximately 24 hours. The roller

drum allows adequate circulation of nutrient and oxygen within the test tube enabling the

bacteria to grow at an optimal rate. After 24 hours, optical densities at 600 nm (O.D.$_{600}$) of the

liquid cultures were obtained using a BioPhotometer (Eppendorf). Each liquid culture was

diluted by a factor of 10 before measuring the O.D.$_{600}$ (to ensure the reading would be in the

linear range of the spectrophotometer) by pipetting 100 µL of each liquid culture and 900 µL of plain LB liquid into each cuvette and mixing to establish a uniform suspension of the bacteria. The spectrophotometer was blanked with plain LB liquid prior to each $O.D._{600}$ reading. The desired $O.D._{600}$ to normalize all isolates was 1.0 ± 0.1 and each isolate was diluted with LB liquid if over that range. No isolates had overnight $O.D._{600}$ readings of less than 1.0.

Preparing 96-well Plates for MICs

Approximately 90 96-well, non-tissue culture treated plates (10 plates for each antibiotic) were prepared in the presence of a nearby lit Bunsen burner. The Bunsen burner was used as a heat barrier to prevent any falling debris or bacteria in the air from contaminating the plates during the preparation. Each plate were prepared by pipetting 200 µL of plain LB liquid into each well then an additional 195.9 µL into the first row, Row A. Then 4.1 µL of a specific antibiotic was pipetted into each well in Row A to achieve a final concentration of 512 µg/mL. The concentration of the antibiotic was then serially diluted by transferring 200 µL from Row A to Row B with mixing, then 200 µL of Row B was transferred to Row C with mixing. This was repeated until Row E where 66 µL of Row E was transferred to Row F with mixing then 66 µL of Row F was transferred to Row G with mixing. Row H did not contain antibiotics to serve as positive controls for bacterial growth. The final antibiotic concentrations for each row on the 96 well plates were as follows: Row A (512 µg/mL), Row B (256 µg/mL), Row C (128 µg/mL), Row D (64 µg/mL), Row E (32 µg/mL), Row F (8 µg/mL), Row G (2 µg/mL), and Row H (0 µg/mL).

Inoculating Plates

To inoculate each plate, 5 μL of each normalized bacterial liquid culture was pipetted in triplicate for each antibiotic to account for biological variability. A Bunsen burner was still used to establish a barrier during inoculation. Positive controls were included on all plates (isolates inoculated into LB with no antibiotics in Row H of each plate). Negative controls were also included on all plates which was a column consisting only plain LB liquid though, in some cases when the antibiotic stock solution was either cloudy or colored, contained the antibiotic also (e.g. rifampin). After inoculation, the plates were wrapped twice with parafilm to prevent evaporation during incubation. Parafilm seals the plates and can sometimes tear upon even mild heating, resulting in small gaps or openings to the plate; therefore each plate was wrapped twice to prevent any small openings that could cause evaporation. To ensure that the bacterial isolates were not limited for oxygen, two plates were incubated together (one wrapped with parafilm and one not wrapped with parafilm). Both plates had the same bacterial isolates and antibiotics used. The O.D.$_{600}$ readings for both plates were similar. The volume of solution for the plate with no parafilm did decrease slightly in some wells. Thus the parafilm prevented the loss of solution volume and did not limit oxygen access to the bacterial isolates. A decrease in any well volume would affect the O.D.$_{600}$ reading during the analysis step by increasing the actual O.D.$_{600}$ reading due to concentrating the bacterial isolates. Finally, the 96-well plates were incubated for approximately 24-48 hours at 37°C without shaking.

Reading the Plates

After the incubation period, the plates were removed from the incubator and stripped of the parafilm. The plates were then read using a Sunrise plate reader (Tecan), which measured the O.D.$_{600}$ of the culture in each well. Each well was scanned 3 times by the machine and the average of each set of readings was given as the output.

To determine the MIC of each antibiotic on each strain, 96-well plates were examined for observable growth. Wells lacking observable growth showed O.D.$_{600}$ readings between 0 and 0.16 (due to the variation in the background and media and because the spectrophotometer did not allow for blanking the readings) while those showing observable growth had readings above this level. Therefore, we chose 0.16 as the baseline to determine the minimum inhibitory concentration for each replicate in each antibiotic. For each sample, the data were examined for the antibiotic concentration at which the O.D.$_{600}$ fell below or equally to 0.16 and this was taken as the MIC for that sample. Each antibiotic MIC was then plotted using GraphPad Prism 5 (graphing software).

Onion Infection Model Assay

Half of the isolates collected by the group of students were also tested in the onion infection assay. The remaining half of the isolates was done with the aid of another post-bac student, Devin McBride, and myself. In preparation, each bacterial isolate was inoculated into a 5 mL plain LB liquid test tube and incubated at 35-37°C on a roller drum for approximately 24 hours.

Preparing the Onion Material

Bags of yellow onions were bought from a Kroger grocery store (200 New Albany Plaza, New Albany, IN 47150 and 3165 S 2nd St, Louisville, KY 40208) for this assay. The skin of the onion was removed including the outer layer. The gloves being worn, the onions, and the bench top were sprayed with 70% ethanol for sterilization. This method was described in Jacobs *et al.*

[18]. The onions were cut in quarters with a kitchen knife, which had been disinfected with 70% ethanol. The layers were then peeled off of one another and cut into approximately 1 inch sections. Only healthy onion peels, indicated by no markings on the peels, were used. The onion peels were placed in an acrylic bin lined with absorbent cloth dampened with sterile water to prevent desiccation of the onion peels. The center of each onion peel was gently pierced with a P200 micropipette tip to serve as the inoculation site.

<u>*Inoculation and Infection Assay*</u>

Each onion peel was inoculated with 5 µL of a designated bacterial liquid culture. Each bacterial isolate were inoculated into three adjacent onion peels to produce triplicates. The acrylic bin was covered with Saran wrap or aluminum foil and incubated at 37°C for approximately 48 hours as described in Jacobs *et al* [18]. The positive control was *Burkholderia cepacia* strain ATCC 25416 (a known onion pathogen) [18], and the negative controls were *Pseudomonas* sp. or no bacterial inoculation. The onion peels were photographed every 24 hours. The data obtained were analyzed by comparing pictures of the onion peels before incubation and at the end of the incubation period (**Supplementary Figure 3**) and rated on a scale of 0 (no maceration) to 3 (massive tissue destruction) based on published protocols [18].

Lettuce Infection Model Assay

<u>*Preparing the Lettuce Material*</u>

Bags of romaine lettuce were bought from a Kroger grocery store (3165 S 2nd St, Louisville, KY 40208). All leaves were detached from healthy-looking cores and discarded since the core served as the infection site. The lettuce cores were then washed with 0.1% bleach and

rinsed with deionized water twice. The cores were then placed in a bin that has been lined with

absorbent cloth soaked in 10 mM magnesium sulfate as described in the Starkey and Laurence

protocol [37]. The magnesium sulfate was used to provide humidity within the bin and prevent

the lettuce cores from desiccating.

Inoculation and Infection Assay

Triplicates were made for each bacterial isolate. Each core was inoculated in the center

with a designated isolate, from the isolate's LB agar plate, using a toothpick. The positive control

was inoculation with *Pseudomonas aeruginosa*, a known lettuce pathogen [37], and the negative

control was not inoculated with a bacterial isolate. The bin was then covered with Saran Wrap to

further slow the lettuce cores from drying out. The bin was then incubated at 30°C for five days.

Throughout the course of the incubation period, the absorbent cloth was kept damp with

magnesium sulfate and the lettuce cores were photographed each day and six hours later each

day for five days. The data obtained were analyzed by comparing pictures of the lettuce core

before incubation and at the end of the incubation period (**Supplementary Figure 4**). Rating

based on maceration level was done by assigning the negative control a score of 0, the positive

control a score of 3, and any intermediate maceration in-between the two mentioned scores was

determined subjectively.

Fungal and Bacterial Interaction Assay

Making Yeast Peptone Dextrose (YPD) Agar Plates

YPD plates were used in this experiment to allow the growth of fungi on agar plates.

YPD plates were made by dissolving 10 g yeast extract, 20 g bacterial peptone, 20 g glucose or

dextrose, and 15 g bacto agar into 1 L of ultrapure water. The medium was then mixed

thoroughly, autoclaved, and placed in a 55°C water bath to cool. Then the medium was poured

into petri dishes where it was left to cool on the bench top. Finally after the medium had

solidified, the plates were flipped and left for overnight drying.

Inoculation of Fungi and Bacterial Isolates

The fungi used in this study were *Aspergillus fumigatus* (ATCC B5852) and *Candida*

albicans (ATCC 23R). The inoculation process involved making three long streaks, which were

evenly spaced, of one of the fungi on a standard 100 millimeter YPD agar plate. Then three short

streaks of a designated bacterial isolate were made to be perpendicular to the three long fungus

streaks using sterile applicators between streaks; therefore it would appear that there were three

intersections of the streaks made representing triplicates of the bacteria isolate (for example, see

Supplementary Figure 5). This method was done for all isolates and the overall procedure was

repeated for the other fungus. The plates were then incubated for approximately 24 hours at

37°C. The procedure was then performed again but this time the three long streaks were

streaked from a bacterial isolate and the three short perpendicular streaks were streaked from a

fungus. This was done for all bacterial isolates. The two fungi were also plated individually to

serve as positive controls. Positive controls for all of the isolates were also streaked on separate

plates.

Analyzing the Interactions

After the incubation period, the plates were examined to see the interaction between the

fungi and the bacteria. Controls were used to compare the interaction to see which microbe (i.e.

fungus or bacterium) outcompeted or inhibited the other since some bacteria looked similar to

one of the fungi. The interactions between microbes were determined by closely examining the

intersection between the streaks of two different microbes. Normally when microbes grow after being streaked on a petri dish, they grow in a solid line filling the indention of the streak. Upon examining the intersection, if the fungal streak was not disrupted by the bacterial streak, then the fungus outcompeted or inhibited the bacteria. If the bacterial streak was not disrupted by the fungal streak, then the bacterium outcompeted or inhibited the fungus.

Antibiotic Single Carbon Source Assay

Making Antibiotic Single Carbon Source (Ab SCS) Plates

Ab SCS plates were made by dissolving 40 mM ammonium sulfate, 20 mM monopotassium phosphate, 2 mM magnesium sulfate heptahydrate, 0.040 mM sodium EDTA, 0.016 mM zinc sulfate heptahydrate, 0.031 mM calcium chloride dihydrate, 0.01 mM iron sulfate heptahydrate, 0.005 mM manganese chloride tetrahydrate, 0.02 mM boric acid, 0.002 mM sodium molybdate dihydrate, 0.001 mM copper sulfate pentahydrate, 0.001 mM cobalt chloride hexahydrate, and 0.0006 mM potassium iodide into 1L of ultrapure water [9]. Some chemical ingredients were unable to be accurately measured out on the scale due to the scale's ability to measure only to 1 mg; therefore any ingredient under 1 mg were measured out by dissolving a single crystal of the certain compound or just enough that was below 1 mg. The pH of the solution was adjusted to approximately 5.5 using hydrochloric acid then the mixture was sterilized through a 0.22 μm filter into two 500 mL glass bottles. Then 7.5 g of plain Lennox LB agar powder was dissolved into each bottle, the medium was autoclaved, and it was placed in a 55°C water bath to cool. Once cooled, 0.5 g of a specific antibiotic was dissolved into one 500 mL bottle. The medium was poured out into plates and cooled on the lab bench top until the agar

had solidified. The plates were then flipped for overnight drying. It should be noted that only 0.1 g of colistin was added due to availability and cost of colistin.

Streaking the Ab SCS Plates

Plates were divided into eight regions. Eight isolates were plated in replicate onto three individual plates (for triplicates) and the process was repeated until all 35 isolates were streaked onto three plates each. *Escherichia coli* K12 was also plated on each of the nine Ab SCS plate as a negative control to ensure the media were made correctly (*E. coli* K12 is susceptible to many antibiotics). The plates were then incubated under 37°C for approximately 24 hours.

Results

Discovery of Antibiotic Resistant Bacterial Isolates

Approximately 162 bacterial isolates were collected for this project (*i.e.* 38 from animals/fungi, 44 from water, 35 from plants, and 45 from soils) but only approximately 138 of the isolates could be re-grown from freezer stocks and appeared to maintain their resistance to colistin or tobramycin. The original goal of the project was to identify specific pathogens which were resistant to either carbenicillin and irgasan (for *Stenotrophomonas maltophilia*) or erthryomycin and irgasan (for *Burkholderia cepacia* complex strains), so the ability of these isolates to resist these combinations was tested. Out of these 138 isolates, only 12 were resistant to a combination of carbenicillin and irgasan, 24 were resistant to a combination of erythromycin and irgasan, and 8 were resistant to all five antibiotics as shown in **Figure 1**. These results showed that majority of the isolates are resistant to colistin and tobramycin with a few isolates

resistant to other antibiotic combinations. If tested with single antibiotics (i.e. just carbenicillin or just irgasan), the isolates may have shown a greater response in resistance.
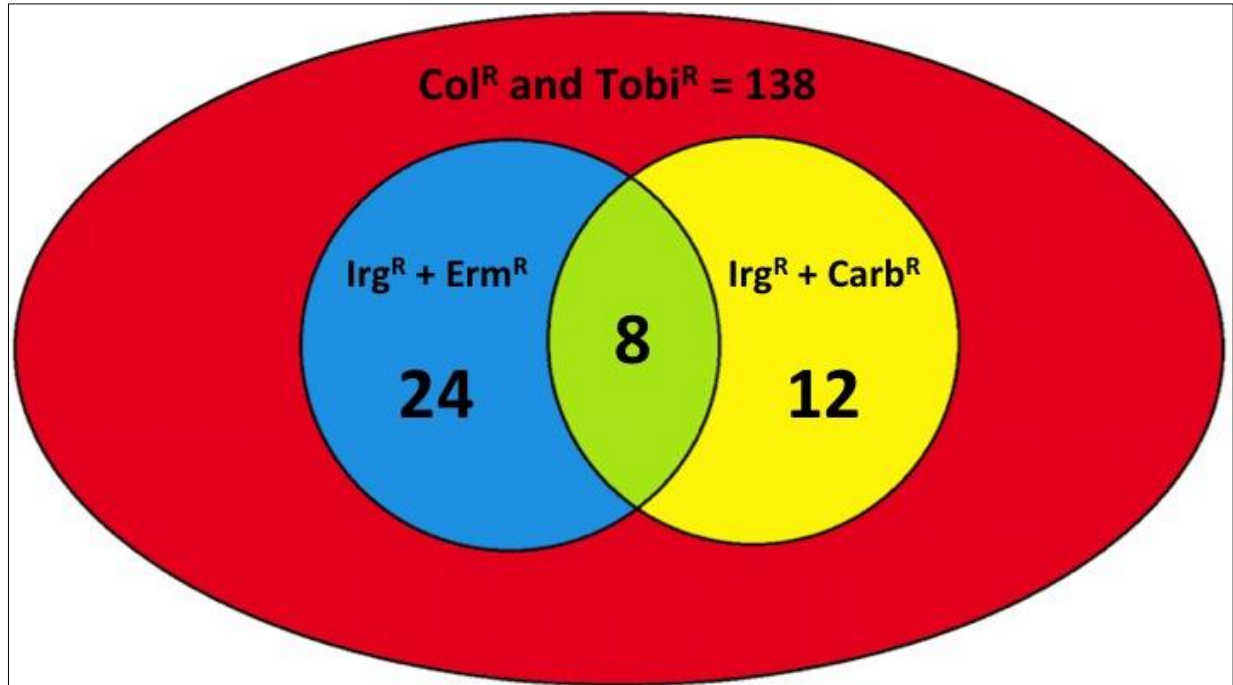

Figure 1. A Representation of Antibiotic Resistant Isolates. The red portion represents isolates that were resistant to the antibiotics colistin (Col^R) and tobramycin (Tobi^R). The blue portion represents isolates that were also resistant to a combination of irgasan (Irg^R) and erythromycin (Erm^R). The yellow portion represents isolates that were also resistant to a combination of irgasan and carbenicillin (Carb^R). The green portion represents isolates that were resistant to all five antibiotics.

Bacterial Species/Genus Identification

The genus/species for each cultured isolates was determined by amplification of the 16S rRNA gene and comparison to online databases. For most of the isolates, the e-value from GenBank (a national gene and protein database) was 0 indicating a strong similarity to known isolates. Similarly, the results from the RDP (Ribosomal Database Project, only contains 16S gene sequences) show a strong indication of a single genera or species associated with each isolate (*i.e.* most isolates had a 100% confidence level). The genus identification of some isolates were slightly unclear due to having a low RDP confidence level (CL) such as *Aminobacterium*

(CL = 33%), Unclassified *Microbacteriacea* (CL = 46%) and *Falsibacillus* (CL = 30%). The 138

isolates represented 27 different genera in which 10 of the genera are known to contain human

pathogens [14]. The ten genera that contain pathogenic species are *Aeromonas*, *Bacillus*,

Burkholderia, *Enterococcus*, *Morganella*, *Providencia*, *Pseudomonas, Rhodococcus*, *Serratia*,

and *Stenotrophomonas*.

The most predominant genus present in all four mentioned types of environments was

Serratia, a Gram-negative bacterium (**Table 2**). Also found in high abundance were *Bacillus*

species, *Spingobacterium* species, and *Paenibacillus* species. Other genera were found less

frequently. This indicates that there are some species which are more prevalent and might be

considered ecological generalists while others may be more specialists for an environment.

These conclusions should be tempered because of sampling bias in siles, samples, environments,

and due to the specific media that was employed and limited sample number. The resultant

organisms might be of interest as they might be reservoirs of antibiotic resistance. Further, they

may represent rare but emerging opportunistic pathogens in veterinary, agricultural, or human

infections. Thus, further testing was warranted.

Minimum Inhibitory Concentration Assay

The goal of the MIC assays was to assess the representative isolates' resistant profile to

the nine antibiotics. Antibiotics were chosen for this assay based on mode of action and

classification to ensure a broad diversity of antibacterial agents (**Table 1**). The hypothesis was

that many of the isolates would be resistant to antibiotics other than tobramycin and colistin. To

establish whether the isolate collection was resistant only to tobramycin and colistin or broadly

Table 2. Summary of Kentucky Isolates and their isolation sites			
Bacterial Genus	**Number of Isolates**	**Isolated From**	**Gram Stain**
Aeromonas	6	Waters	-
Arthrobacter	3	Soils	+
Bacillus	23	Waters, Soils, Insects, Fungi	+
Brevibacillus	8	Water, Soil, Insects	+
Burkholderia	4	Soils	-
Cellulosimicrobium	1	Soil	+
Chromobacterium	1	Water	-
Chryseobacterium	10	Water, Plants	-
Cloacibacterium	1	Water	-
Elizabethkingia	1	Soil	-
Empedobacter	1	Water	-
Enterococcus	2	Animal feces	+
Flavobacterium	1	Water	-
Leucobacter	1	Plant	+
Microbacterium	8	Water, Plant, Soils	+
Morganella	2	Water	-
Myroides	1	Insect	-
Paenibacillus	10	Soils, Waters, Plants	+
Providencia	4	Plants	-
Pseudochrobacterum	2	Soils	-
Pseudomonas	2	Plants	-
Rhodococcus	1	Soil	+
Serratia	31	Plants, Soils, Fungi, Water	-
Sphingobacterium	13	Water, Soil, Insects	-
Stenotrophomonas	1	Soil	-
Total	**138**		

to many antibiotics, each representative isolate were inoculated into 96 well plates containing

serially diluted antibiotics. The antibiotics used in this study were chosen for their breadth in

terms of their mechanism of action and the types of bacteria they are known to inhibit (**Table 1**).

For the genera with an abundance of isolates (*e.g. Bacillus*, *Serratia*, and *Sphingobacterium* -

Table 2), 2-3 representative isolates were used. The results are shown in **Table 3**.

A low MIC reveals that a bacterium is highly susceptible to the antibiotic and a high MIC could be interpreted as a low sensitivity or complete resistance against the antibiotic. Most isolates that were subjected to colistin, penicillin, tobramycin, or trimethoprim were highly resistant to these antibiotics with MICs greater than 512 µg/mL (indicated with "**" in **Table 3**), which indicates that they are resistant to all concentrations tested. Most of the isolates that were subjected with chloramphenicol, kanamycin, and nalidixic acid had many MIC values in the low to intermediate range (*i.e.* values in the range of 2-512 µg/mL). Most isolates that were subjected with rifampin and tetracycline had low MIC values (*i.e.* values in less than 64 µg/mL), which indicate little resistance to these antibiotics in these natural isolates.

Table 3. Minimum inhibitory concentrations of 36 representative isolates for nine antibiotics.									
	Antibiotics (µg/mL)								
Bacterial Isolates	Chloramphenicol	Colisitin	Kanamycin	Nalidixic Acid	Penicillin	Rifampin	Tetracycline	Tobramycin	Trimethoprim
Aeromonas caviae	2	**	2	2	2	2	2	32	32
Aeromonas hydrophila	8	*512	256	128	**	8	2	32	**
Aminobacterium	64	512	512	256	**	8	64	64	**
Arthrobacter nitroguajacolicus	32	512	*512	64	**	256	*128	*64	2
Bacillus cereus	*128	**	64	64	**	32	2	32	**
Bacillus thuringiensis, -anthracis, -cereus	8	512	128	128	**	8	2	32	**
Bacillus thuringiensis, -cereus, -toyonensis	8	**	*512	32	**	2	8	64	**
Brevibacillus laterosporus	512	512	32	256	2	2	8	8	2
Brevibacillus reuszeri, -choshinensis, -brevis	8	**	*512	256	*256	2	*8	*256	**
Burkholderia cepacia	8	*512	*512	128	**	8	8	256	**
Cellulosimicrobium funkei, -cellulans	8	256	**	256	32	2	2	**	32
Chromobacterium aquaticum	32	**	64	128	**	64	2	64	8
Chryseobacterium letacus	8	512	32	64	512	2	2	32	**
Chryseobacterium stagni, -gambrini	128	**	*64	64	**	256	*128	*64	*2
Cloacibacterium sp./unknown	8	*256	8	256	8	32	8	8	2
Elizabethkingia meningoseptica, -anophelis	64	**	256	256	512	2	128	512	32
Empedobacter brevis	32	*512	*256	*256	*512	8	*32	**	32
Enterococcus casseliflavus, -gallinarum	32	512	2	2	2	2	2	8	2
Falsibacillus	32	**	*512	128	*512	2	64	256	8
Flavobacterium sp.	32	**	512	128	32	2	8	512	**
Leucobacter chromiireducens	*256	256	*512	256	512	2	8	**	32
Microbacterium arborescens	64	**	*256	**	*32	2	*8	*64	**
Morganella morganii	8	**	*64	32	**	2	2	32	**
Myroides odoratus	256	**	**	128	512	32	2	**	256
Paenibacillus alvei	2	*512	*512	32	**	2	*8	*256	*512
Paenibacillus apiarius	32	*512	*512	8	8	2	2	128	32
Providencia	512	**	64	*256	**	256	64	64	8
Pseudochrobactrum asaccharolyticum, -saccharolyticum	8	512	128	64	512	64	32	64	128
Pseudomonas protegens	8	**	**	128	512	2	32	**	8
Rhodococcus equi	8	**	*512	64	**	8	*64	*256	*8
Serratia marcescens	128	**	512	256	**	512	64	64	8
Serratia marcescens	32	**	512	64	**	256	64	64	8
Serratia marcescens, -nematodiphila	*64	**	512	64	**	*256	64	128	8
Sphingobacterium faecium	32	256	256	32	**	2	2	128	**
Sphingobacterium siyangensis, -multivorum	**	**	**	128	*64	2	*64	**	*2
*One or two trials had MIC higher than 512 µg/mL									
**All three tirals had MIC higher than 512 µg/mL									
***Values from the table represent the median value from triplicate samples									

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The dynamics of the interaction between each representative bacterial strain and antibiotic is illustrated in **Figures 2-10**. For each antibiotic, seven strains were analyzed together in the same plot to avoid confusion and were combined alphabetically. Each graph within each figure tends to show a consistent pattern for most of the isolates for each antibiotic (e.g. most isolates in **Figure 2** show a negative relationship between growth and increasing antibiotic concentration). The antibiotics chloramphenicol, nalidixic acid, and rifampin showed drastic decreases in $O.D._{600}$ levels for most isolates as antibiotic concentration increased especially at low concentrations. The antibiotics colistin, kanamycin, penicillin, tetracycline, and tobramycin showed small decreases growth for most isolates but also a small increase in $O.D._{600}$ level for a few isolates as antibiotic concentration increased. Trimethoprim showed a unique set of MIC results in that more isolates than anticipated had an increasing $O.D._{600}$ level as antibiotic concentration increased (**Figure 10**).

Plant Model Assay

Bacteria in the soil can be beneficial to plants by protecting them against pathogens or by fixing nitrogen from the air which is necessary for plant growth. Bacteria can also be plant pathogens and can infect the roots, stems, and leaves of plants. The goal of this assay was to determine the pathogenicity of the representative isolates towards onions and lettuce. The hypothesis of this assay was that some of the isolates were pathogenic to these two plant models. To test the pathogenicity of the representative panel isolates for the ability to cause pathogenesis in two plant models – onion bulb and lettuce leaves, the isolates were inoculated into sterile onion peels and lettuce cores as described in the *Materials and Methods* section according to published protocols [18, 37].
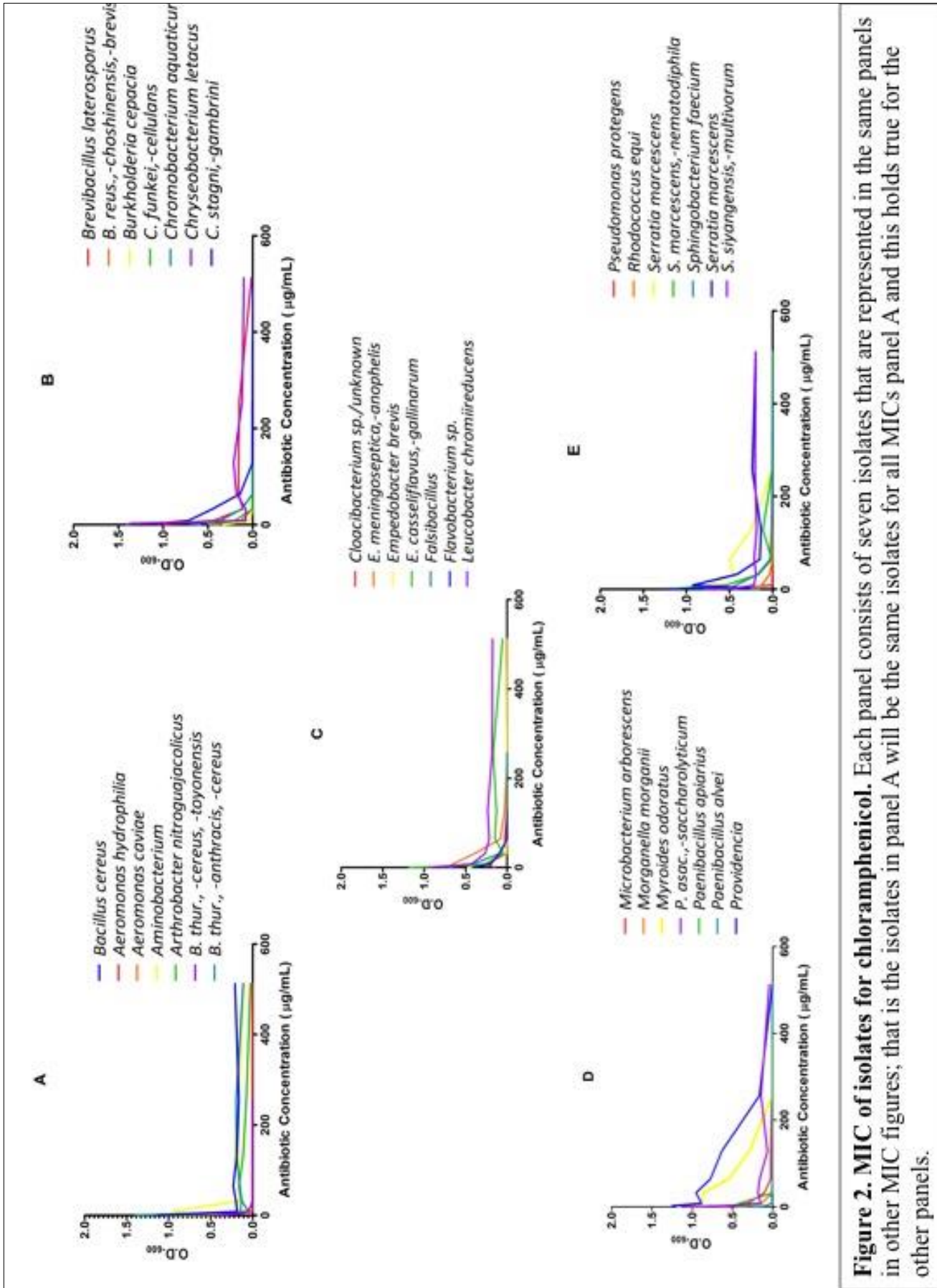
Figure 2. MIC of isolates for chloramphenicol. Each panel consists of seven isolates that are represented in the same panels in other MIC figures; that is the isolates in panel A will be the same isolates for all MICs panel A and this holds true for the other panels.
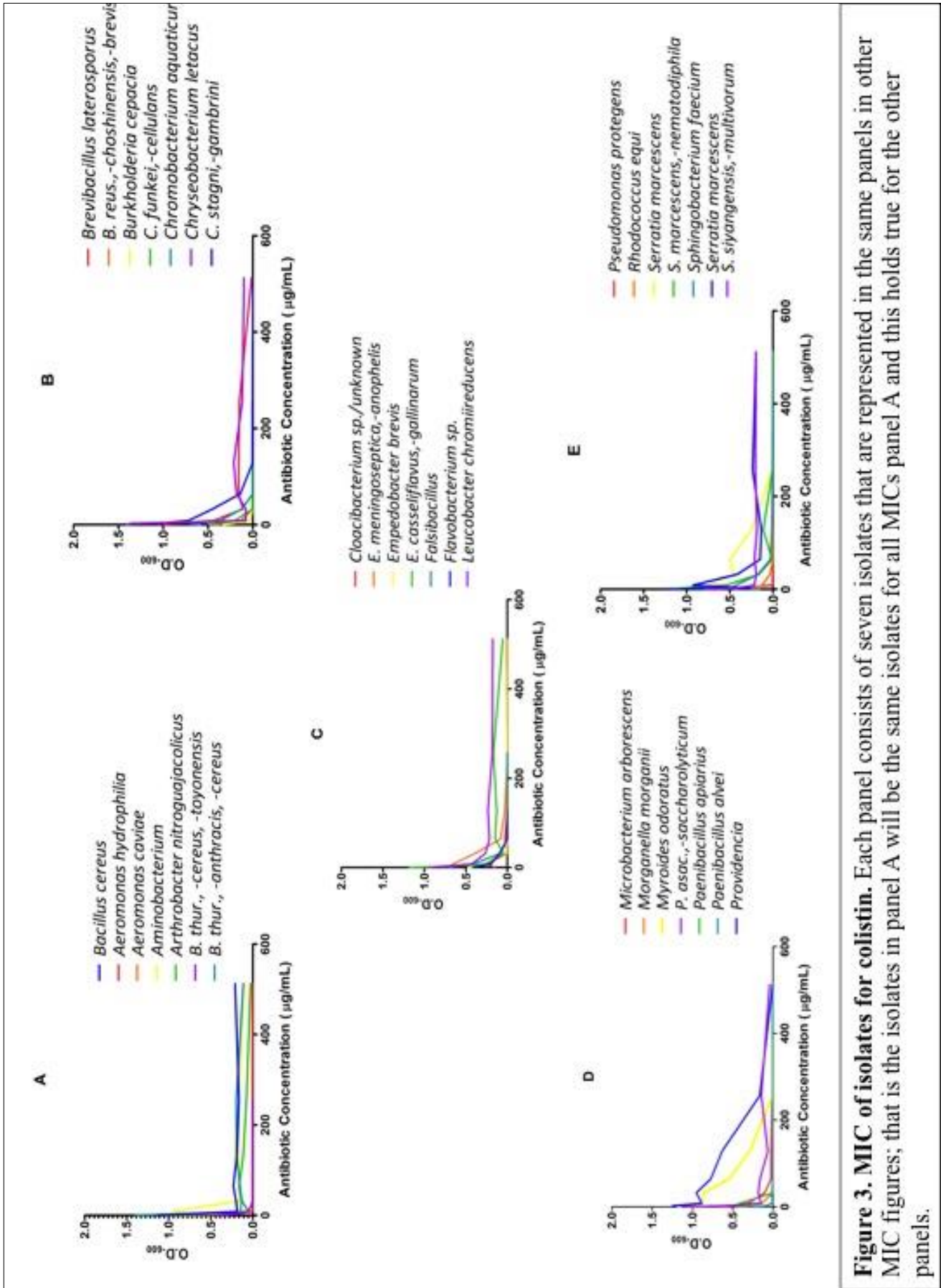
A

- Bacillus cereus
- Aeromonas hydrophilia
- Aeromonas caviae
- Aminobacterium
- Arthrobacter nitroguajacolicus
- B. thur., -cereus, -toyonensis
- B. thur., -anthracis, -cereus

B

- Brevibacillus laterosporus
- B. reus., -choshinensis, -brevis
- Burkholderia cepacia
- C. funkei, -cellulans
- Chromobacterium aquaticum
- Chryseobacterium letacus
- C. stagni, -gambrini

C

- Cloacibacterium sp./unknown
- E. meningoseptica, -anophelis
- Empedobacter brevis
- E. casseliflavus, -gallinarum
- Falsibacillus
- Flavobacterium sp.
- Leucobacter chromiireducens

D

- Microbacterium arborescens
- Morganella morganii
- Myroides odoratus
- P. asac., -saccharolyticum
- Paenibacillus apiarius
- Paenibacillus alvei
- Providencia

E

- Pseudomonas protegens
- Rhodococcus equi
- Serratia marcescens
- S. marcescens, -nematodiphila
- Sphingobacterium faecium
- Serratia marcescens
- S. siyangensis, -multivorum

Figure 3. MIC of isolates for colistin. Each panel consists of seven isolates that are represented in the same panels in other MIC figures; that is the isolates in panel A will be the same isolates for all MICs panel A and this holds true for the other panels.
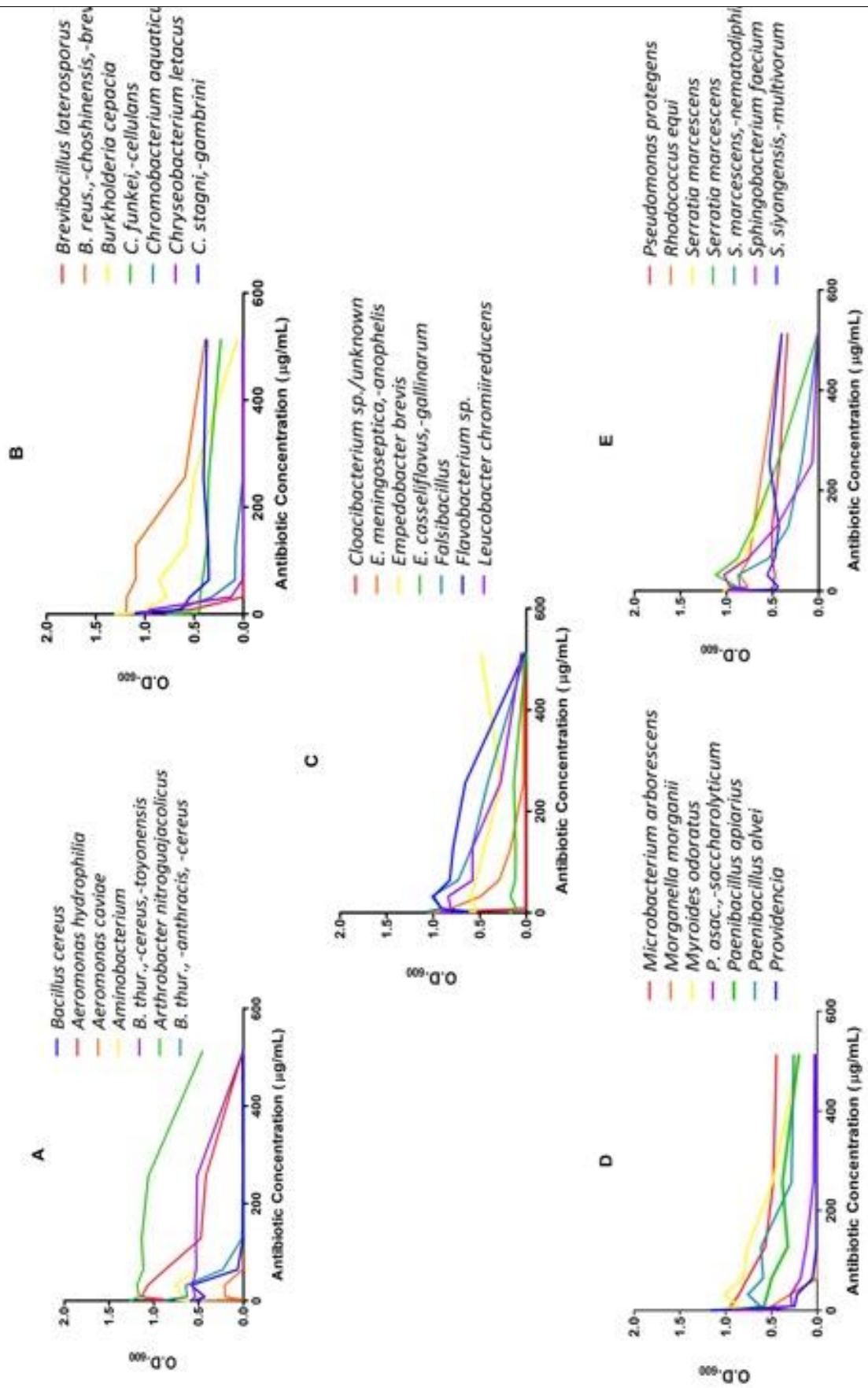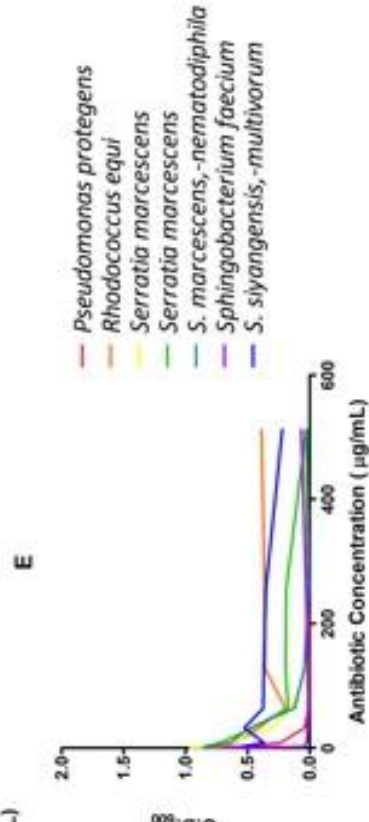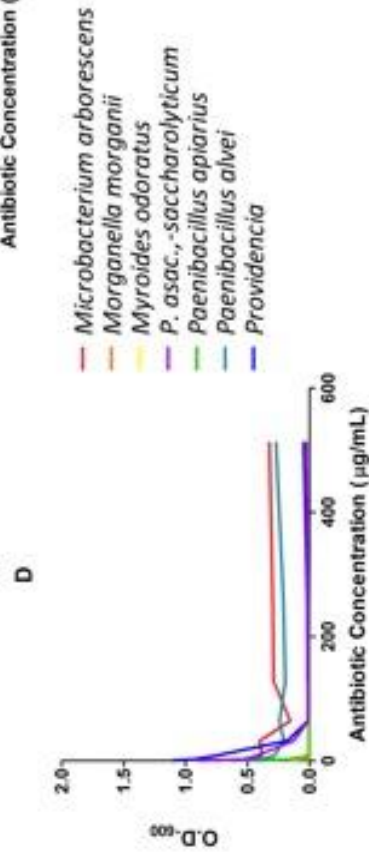
Figure 4. MIC of isolates for kanamycin. Each panel consists of seven isolates that are represented in the same panels in other MIC figures; that is the isolates in panel A will be the same isolates for all MICs panel A and this holds true for the other panels.
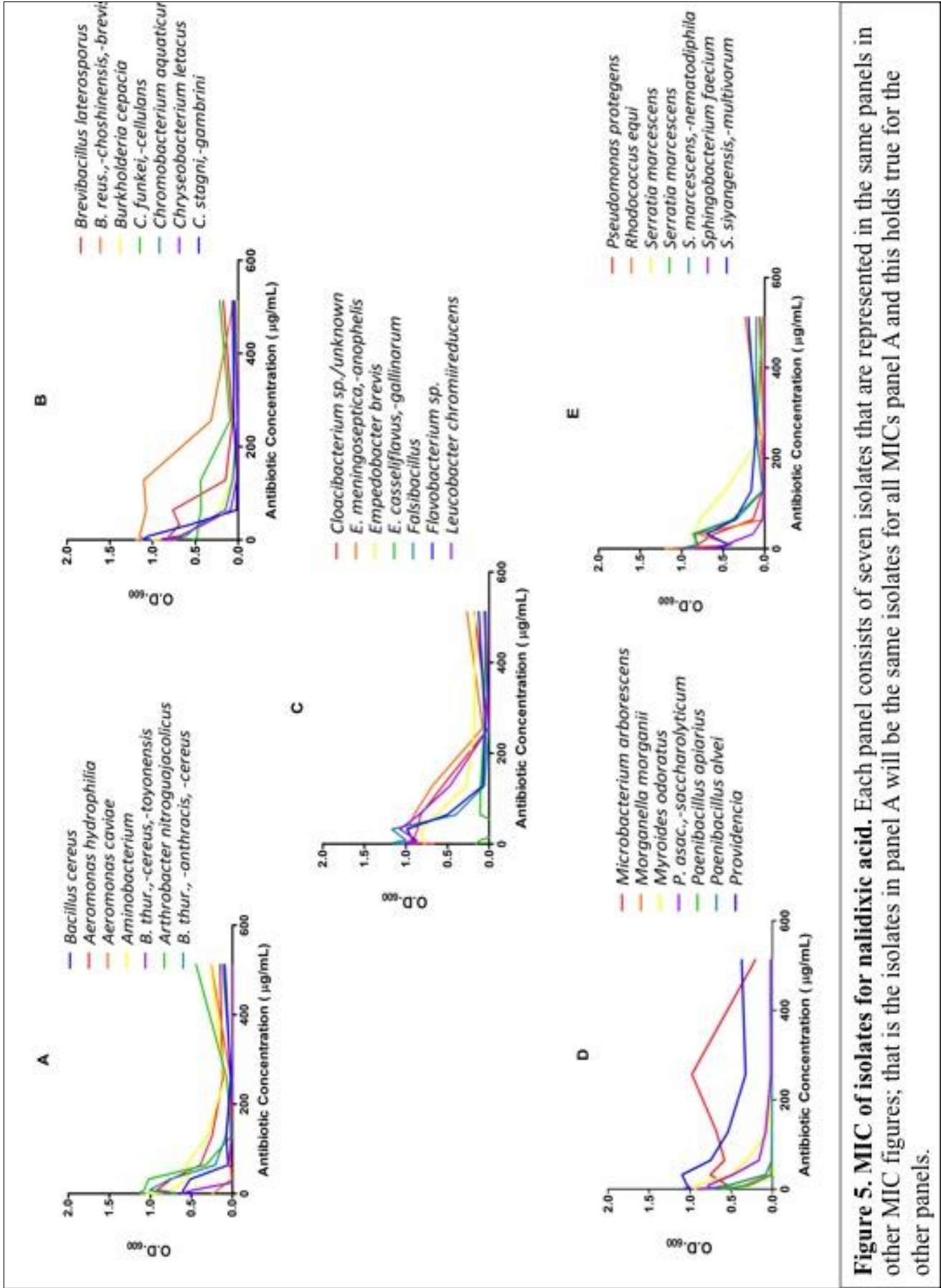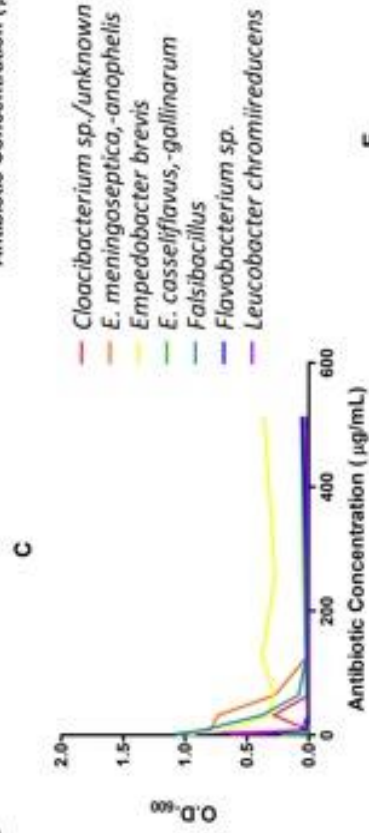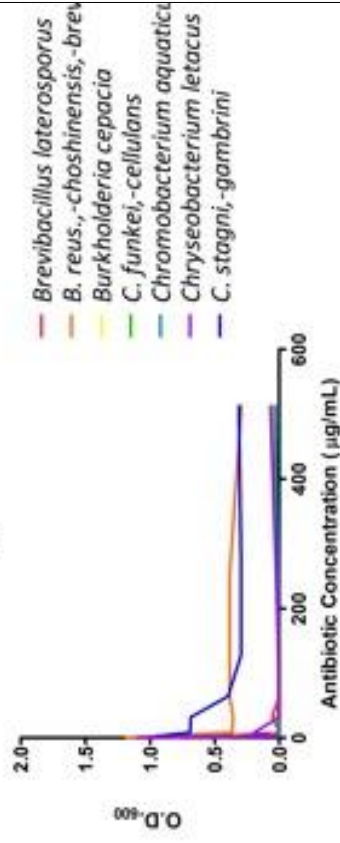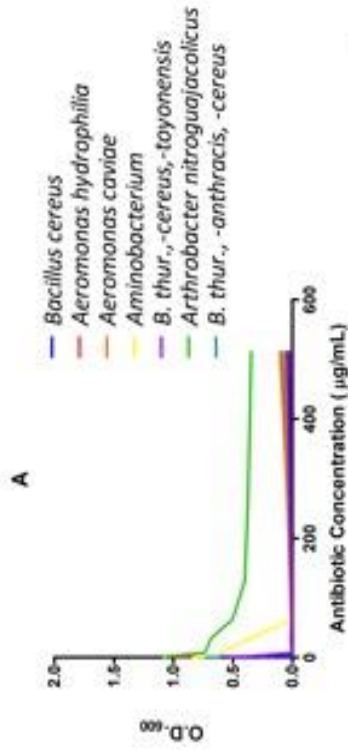
Figure 5. MIC of isolates for nalidixic acid. Each panel consists of seven isolates that are represented in the same panels in other MIC figures; that is the isolates in panel A will be the same isolates for all MICs panel A and this holds true for the other panels.
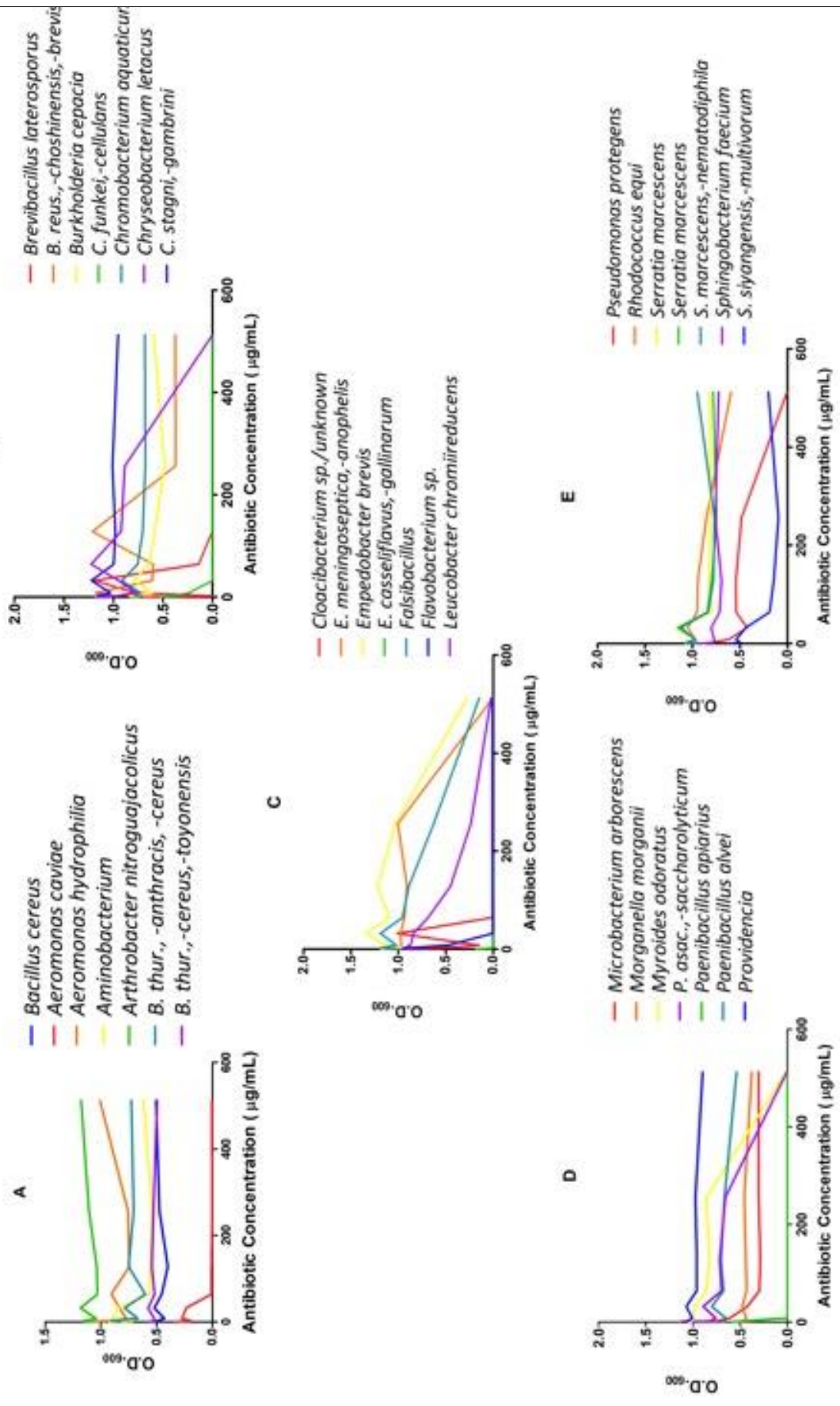
Figure 6. MIC of isolates for penicillin. Each panel consists of seven isolates that are represented in the same panels in other MIC figures; that is the isolates in panel A will be the same isolates for all MICs panel A and this holds true for the other panels.
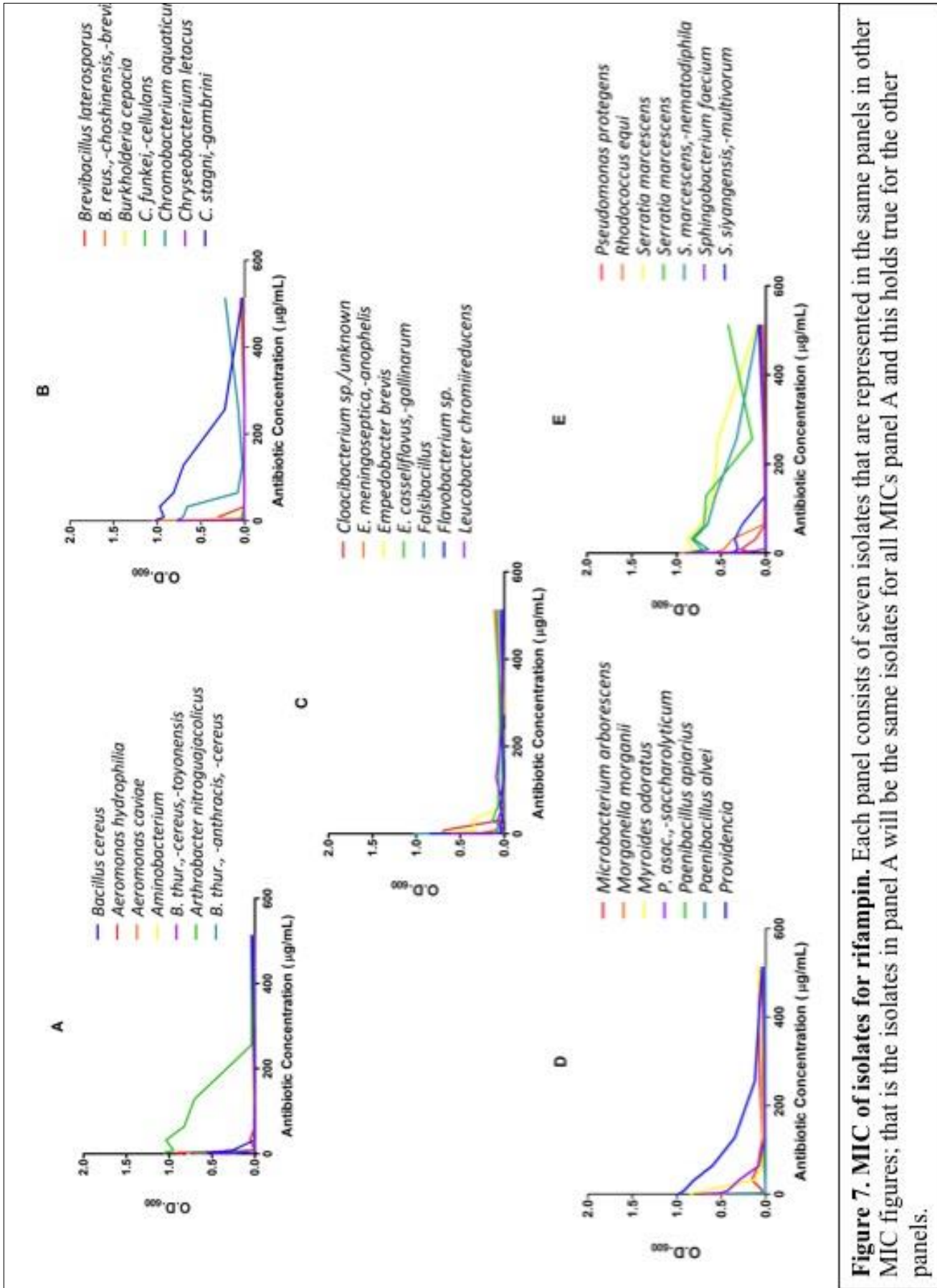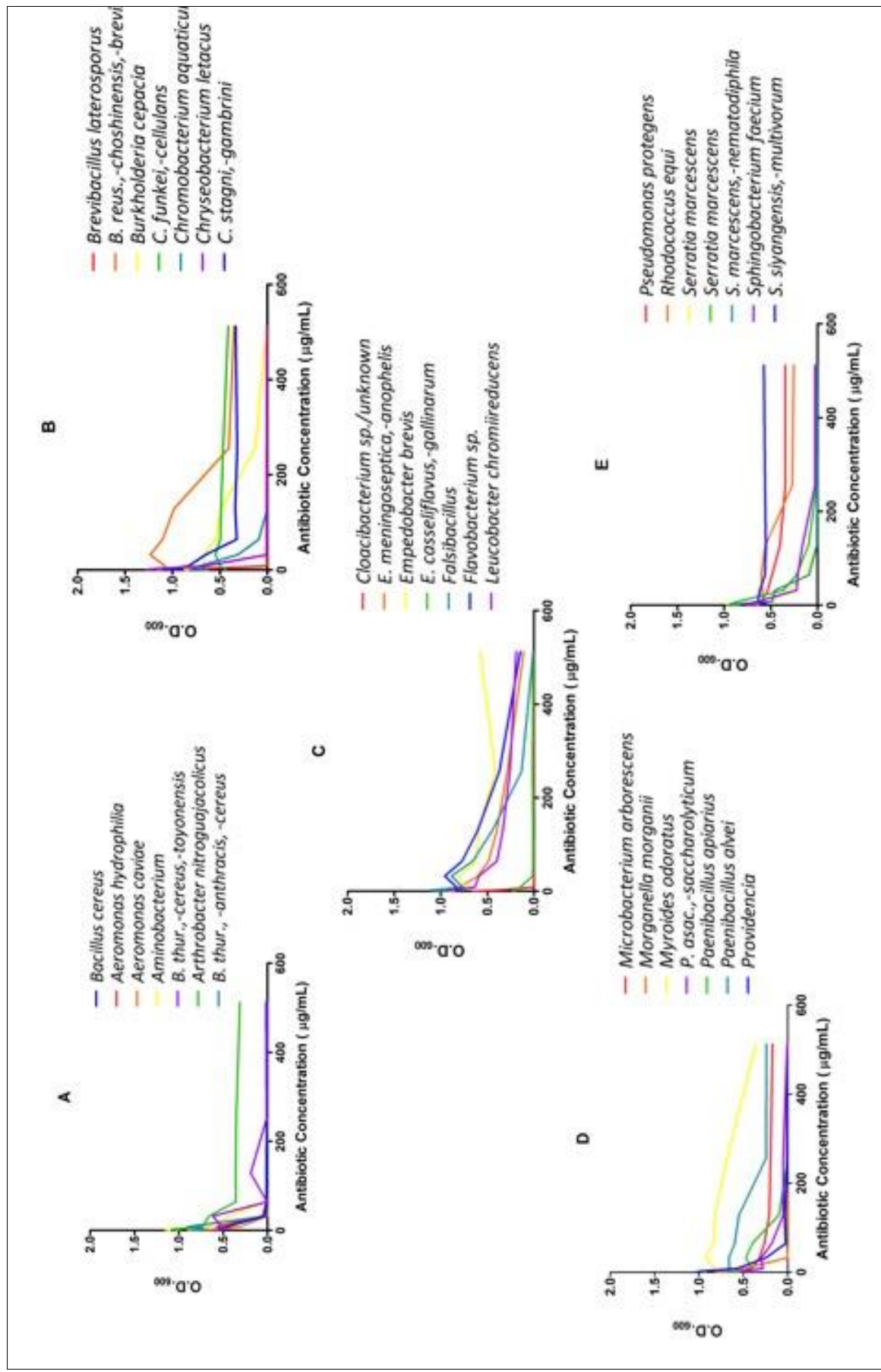
Figure 7. MIC of isolates for rifampin. Each panel consists of seven isolates that are represented in the same panels in other MIC figures; that is the isolates in panel A will be the same isolates for all MICs panel A and this holds true for the other panels.

Figure 8. MIC of isolates for tetracycline. Each panel consists of seven isolates that are represented in the same panels in other MIC figures; that is the isolates in panel A will be the same isolates for all MICs panel A and this holds true for the other panels.
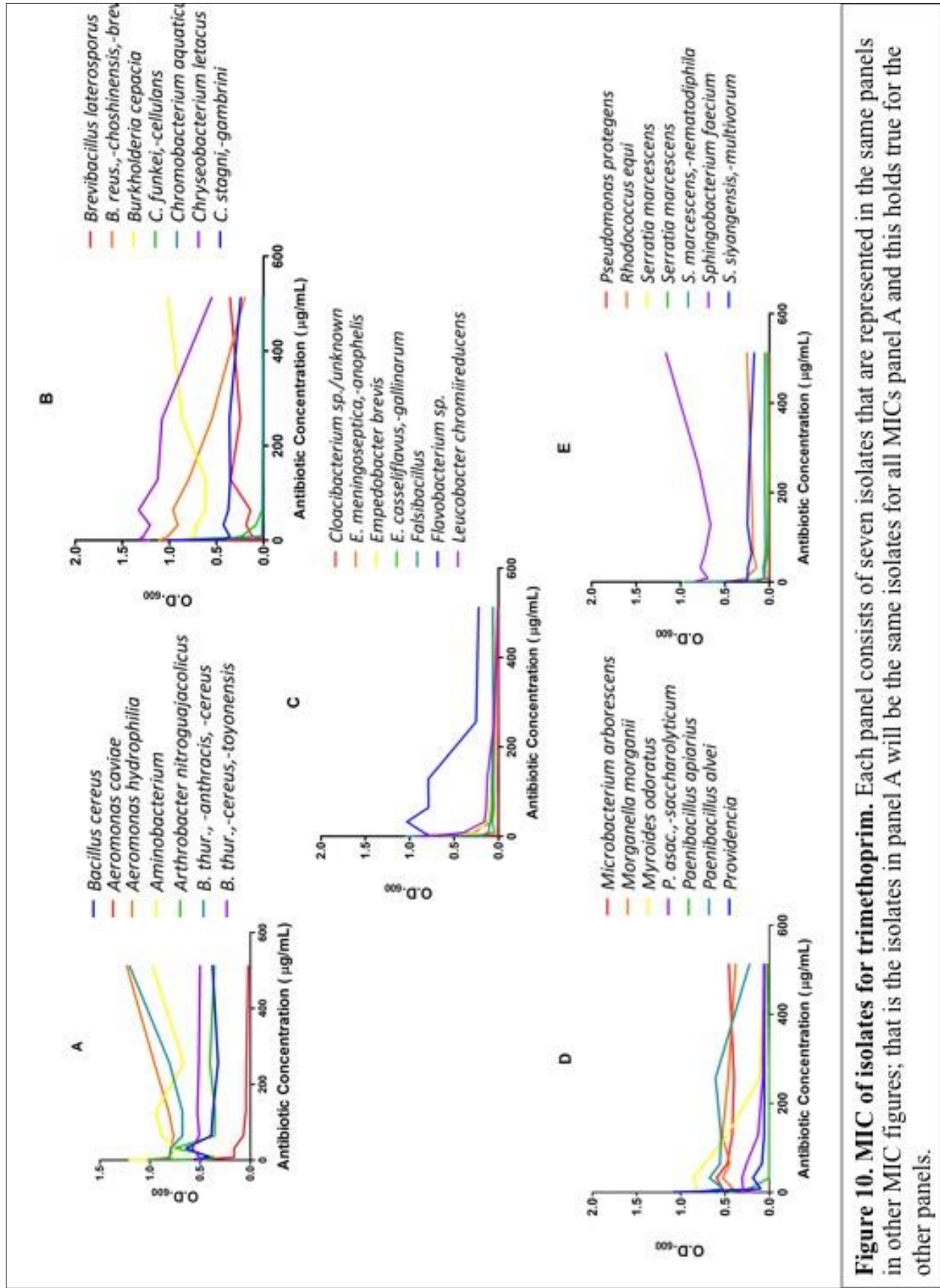
Figure 9. MIC of isolates for tobramycin. Each panel consists of seven isolates that are represented in the same panels in other MIC figures; that is the isolates in panel A will be the same isolates for all MICs panel A and this holds true for the other panels.

Figure 10. MIC of isolates for trimethoprim. Each panel consists of seven isolates that are represented in the same panels in other MIC figures; that is the isolates in panel A will be the same isolates for all MICs panel A and this holds true for the other panels.

Tissue damage was assessed qualitatively and by comparing to control inoculations. In the onion maceration model, surprisingly only two strains, *Myroides odoratus* and *Sphingobacterium faecium*, showed some form of onion tissue maceration (**Figure 11**). In the lettuce maceration model, five genera of the twenty-seven showed some form of lettuce tissue maceration though it was not significant (**Figure 11**). The majority of the genera were unable to visibly infect or macerate either plant model revealing that most of these isolates are not pathogens of yellow onions and lettuce. The few that were able to macerate the plants were isolates collected from sources that did not involve plants with the exception of one, *Sphingobacterium faecium*. From this, plant-associated bacteria appear to be less likely to be plant pathogens. With the controls effectively macerating their respective plant model, the data could be interpreted that the plant itself may have been able to defend itself against the bacterial isolates or that the isolates lacked the appropriate cellulases or other enzymes that could lead to tissue destruction. It could also be that some of these isolates could serve a beneficial role to onion or lettuce plants; however, testing this was outside the scope and abilities of our research lab.

Fungal and Bacterial Interaction Assay

The goal of the fungal-bacterial interaction assay was to observe the microbial interaction between the representative isolates and the two fungi, *A. fumigatus* and *C. albicans*. The hypothesis of this assay was that the fungi were capable of outcompeting or inhibiting some of the isolates. To observe if there is a microbial interaction between the fungi and bacteria, both microbes were cross-streaked as described in *Materials and Methods* section and depicted in **Supplementary Figure 5**. The bacterial isolates showed variable responses to the fungi when

Lettuce and Onion Maceration Model

streaked in different orders (*i.e.* first or second). Approximately half of the isolates were unable to outcompete or inhibit the fungus *A. fumigatus* when the fungus was streaked first. When the bacterium was streaked first, only six isolates were unable to outcompete the fungus (**Figure 12**).

To interpret this graph, an example is given: *Falsibacillus* was able to outcompete the *A. fumigatus* when the bacterium was streaked first but unable to outcompete the fungus when the bacterium was streaked second. Approximately more than half of the isolates were able to outcompete the fungus *Candida albicans* regardless of the order of microbial streaking (**Figure 13**). It appeared that in some microbial interactions, the order of placement determines the microbial competitiveness. Of the isolates outcompeted by *A. fumigatus*, most of the isolates were Gram-positive. Of the isolates outcompeted by *C. albicans*, most of the isolates were Gram-negative. This may reveal that some fungi are more target-specific in regards to bacteiral inhibition.

Antibiotic Single Carbon Source Assay

In a study recently published, researchers identified that some soil bacteria could not only tolerate high levels of antibiotics but could consume them as a carbon source [9]. They identified these organisms by plating them on a solid medium containing salts and an antibiotic. To determine whether the isolates collected in this study could consume antibiotics in a similar manner, we struck our isolates on a minimum salts agar containing 0.5 g/L of antibiotic (0.1 g/L for colistin). After growth at 37°C, most isolates did not show the capability to consume most of the antibiotics (**Table 4**).
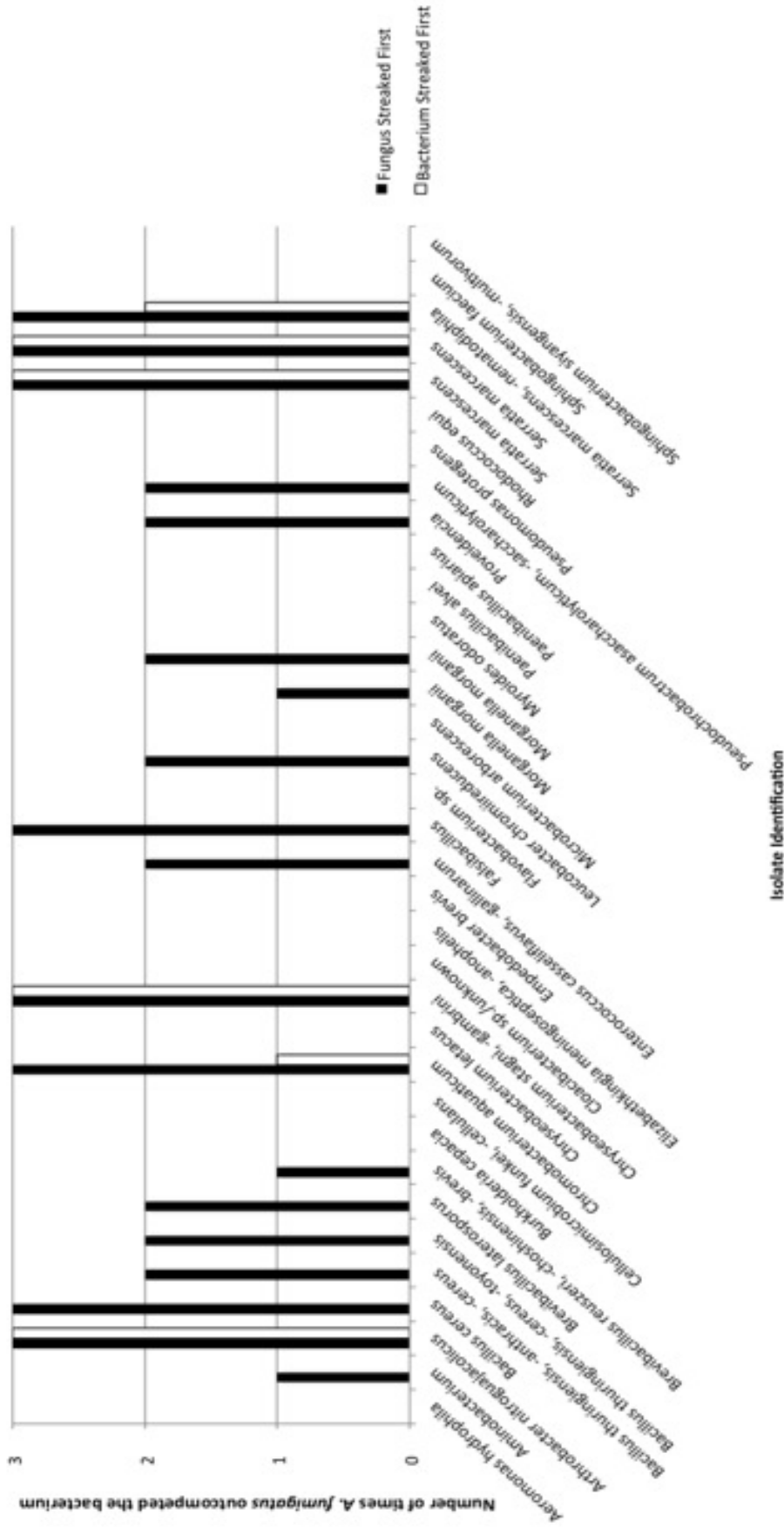
Figure 12. Analysis of cross-streaking between *Aspergillus fumigatus* and the bacterial isolates. Microbial interaction between *A. fumigatus* and the bacterial isolates were observed when the fungus was placed on the YPD agar plate first (solid bars) and when the bacterium was placed first (hollow bars). The number of times the fungus outcompeted or inhibited the bacterium was out of three trials.
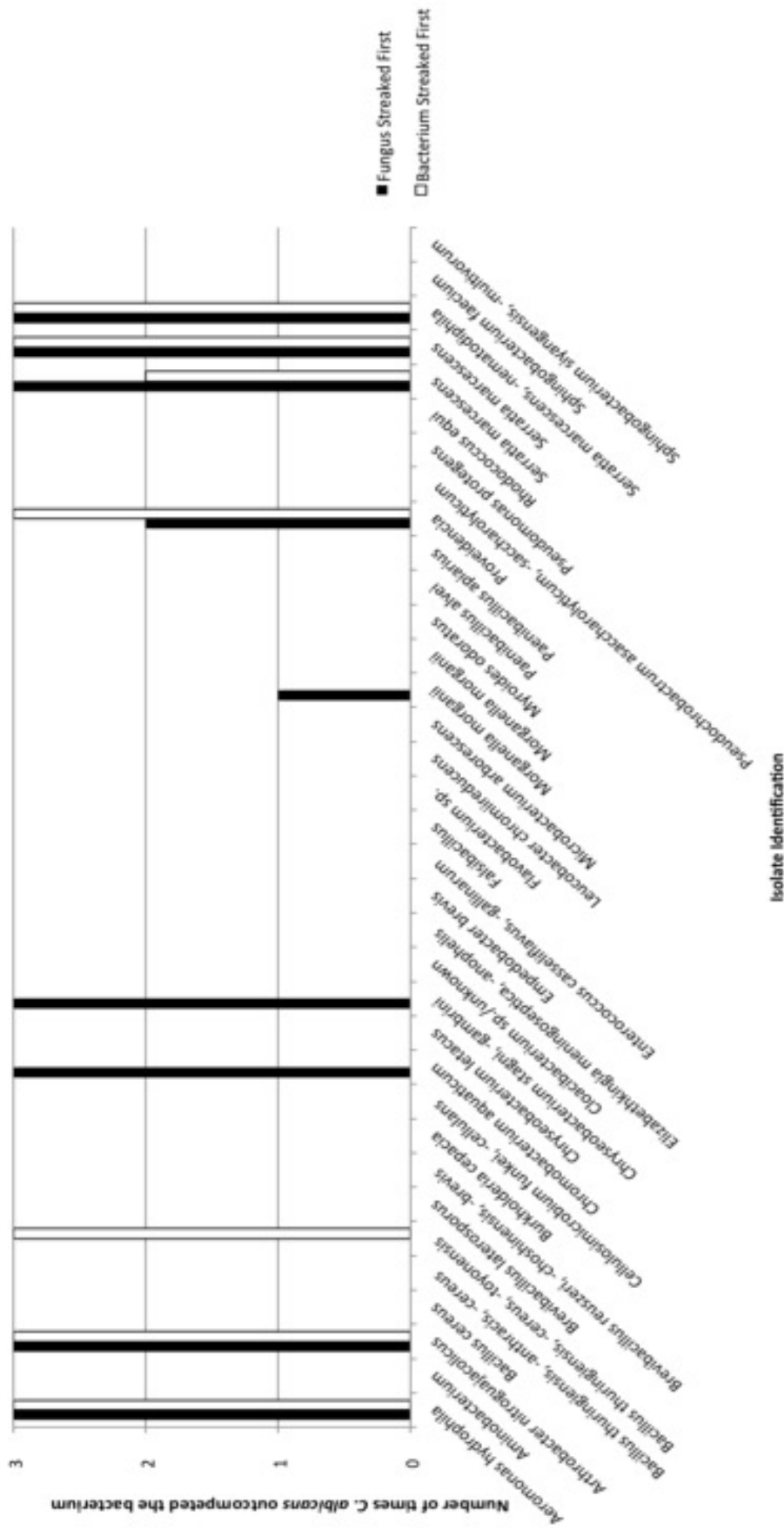
Figure 13. Analysis of cross-streaking between *Candida albicans* and the bacterial isolates. Microbial interaction between *C. albicans* and the bacterial isolates were observed when the fungus was placed on the YPD agar plate first (solid bars) and when the bacterium was placed first (hollow bars). The number of times the fungus outcompeted or inhibited the bacterium was out of three trials.

Colistin was the only antibiotic that could be consumed by most of the isolates (**Table 4**). Kanamycin, penicillin, tobramycin, and trimethoprim were able to be consumed by less than half of the isolates. Chloramphenicol, nalidixic acid, rifampin, and tetracycline were unable to be consumed by any isolate with an exception of one or two isolates. This suggests that most of the isolates that are resistant to the antibiotics tolerate and detoxify these antibiotics but may not break them down for consumption as carbon sources. Since the concentration of colistin used was lower than the desired concentration (*i.e.* 0.10 g/L instead of 0.50 g/L), this may explain the reason for most of the isolates being able to consume this particular antibiotic though the concentration is still high.

Table 4. Analysis of isolates' ability to utilize certain antibiotics as a sole carbon source.

Bacterial Isolates	Antibiotics								
	Chloramphenicol	Colisitin	Kanamycin	Nalidixic Acid	Penicillin	Rifampin	Tetracycline	Tobramycin	Trimethoprim
Aeromonas caviae	x	2	x	x	x	x	x	x	3
Aeromonas hydrophila	x	x	x	3	x	x	x	x	x
Aminobacterium	x	3	2	x	1	x	x	x	3
Arthrobacter nitroguajacolicus	x	2	x	x	3	x	2	x	x
Bacillus cereus	x	3	2	x	3	x	x	x	3
Bacillus thuringiensis, -anthracis, -cereus	x	3	x	x	x	x	x	x	3
Bacillus thuringiensis, -cereus, -toyonensis	x	3	3	x	x	x	x	1	3
Brevibacillus laterosporus	x	x	x	x	x	x	x	x	x
Brevibacillus reuszeri, -choshinensis, -brevis	x	x	3	x	1	x	x	3	2
Burkholderia cepacia	x	3	3	x	x	x	x	3	x
Cellulosimicrobium funkei, -cellulans	x	x	x	x	x	x	x	3	x
Chromobacterium aquaticum	x	3	2	x	x	x	x	x	x
Chryseobacterium letacus	x	3	x	x	x	x	x	x	3
Chryseobacterium stagni, -gambrini	x	3	3	x	3	x	x	3	x
Cloacibacterium sp./unknown	x	x	x	x	x	x	x	x	x
Elizabethkingia meningoseptica, -anophelis	x	3	3	x	x	x	x	3	x
Empedobacter brevis	x	x	3	x	3	x	x	3	x
Enterococcus casseliflavus, -gallinarum	x	3	x	x	1	x	x	x	x
Falsibacillus	x	3	3	x	x	x	x	3	x
Flavobacterium sp.	x	3	x	x	x	x	x	x	3
Leucobacter chromiireducens	x	x	3	x	x	x	x	3	x
Microbacterium arborescens	x	3	x	x	1	x	x	x	3
Morganella morganii	x	3	x	x	1	x	x	x	2
Myroides odoratus	x	3	2	x	x	x	x	3	3
Paenibacillus alvei	x	x	3	x	x	x	x	3	x
Paenibacillus apiarius	x	x	x	x	x	x	x	x	x
Providencia	x	x	x	3	3	x	x	x	3
Pseudochrobactrum asaccharolyticum, -saccharolyticum	x	3	3	x	x	x	x	x	3
Pseudomonas protegens	x	3	3	x	x	x	x	3	x
Rhodococcus equi	x	x	2	x	x	x	x	x	x
Serratia marcescens	x	3	x	x	2	x	x	x	x
Serratia marcescens	x	3	x	x	3	x	x	x	x
Serratia marcescens, -nematodiphila	x	3	x	x	x	x	x	x	x
Sphingobacterium faecium	x	x	2	x	x	x	x	3	x
Sphingobacterium siyangensis, -multivorum	x	3	3	x	x	x	x	3	x

*A number indicates the number of replicates for which there was observed growth on the antibiotic single carbon source plate (out of three trials). An "x" indicates no observed growth from all three trials.

Discussion

Surveying and collecting a small sample size of bacterial isolates from natural environments within Kentucky revealed a great diversity with at least ten genera containing known human pathogenic strains. The 27 genera discovered in this sample contained species resistant to colistin and tobramycin with a few resistant to carbenicillin, erythromycin, and irgasan. Though these bacterial isolates were collected from areas believed to have low human interactions, it can be noted that these resistant isolates are potentially able to transfer antibiotic resistance genes to non-resistant bacteria through means such as conjugation, transformation, and transduction. It should also be noted that even though some of these strains are not common human pathogens, even "non-pathogenic" or commensal bacteria maybe able to cause infections in susceptible hosts. As there are great number of people globally that are immunocompromised (*e.g.* HIV patients, patients undergoing chemotherapy, or patients who have recently had transplants and are chemically immunosuppressed) or have underlying conditions (*e.g.* severe diabetes, cystic fibrosis, severe burns) which tax the immune system, this work could reveal potential pathogens that may be difficult to eradicate. The more that is learned about these strains in advance, the better off the treatment could potentially be for those afflicted by these bacteria.

The 36 representative isolates of the 27 genera showed variable responses to each antibiotic in the MIC assay. The antibiotics that resulted in a high MIC can be inferred as ineffective against the tested isolates. Other antibiotics with intermediate to low MIC can be seen as having some form of efficacy in combating these isolates. Colistin, penicillin, tobramycin, and trimethoprim have shown to be less effective in combating the bacterial isolates. Chloramphenicol, kanamycin, and nalidixic acid were effective against some bacterial strains,

but not others. This data and similar data from other groups will hopefully allow antibiotic usage

to be re-evaluated and increase development of new antibiotics. Re-evaluating the use of

antibiotics involves changing antibiotic dosage or the use of other treatment protocols such as

homeopathic treatments. New antibiotics could be developed by slightly changing the chemical

structure of the antibiotic, which could increase the efficacy, or identifying novel antibiotics

from natural or synthetic sources. It should be noted that the MIC assay was done with only

single antibiotics; testing with a combination of antibiotics could lead to synergistic effects of the

antibiotics.

The bacterial isolates showed little capability of infecting the plant models. This could be

due to the plant's innate ability to fend off the bacterial invasion or a lack of virulence

determinants specific for plants in the bacteria. Plants and bacteria have known symbiotic

relationships, for example nitrogen-fixing bacteria that provide ammonia to plants and receive

carbon compounds in return. When the interaction between the two is pathogenic (parasitic),

plants have developed defense mechanisms against plant pathogenic bacteria through evolution.

One unique mechanism that evolved in plants for protection was the production of specialized

metabolites otherwise known as phytochemicals that convey antimicrobial properties [30]. In the

detection of a bacterial invasion (inoculation sites), the plant may release the phytochemical to

defend itself. On the other hand, the isolates may not be pathogenic against the two plant models

and could be neutral or plant beneficial bacteria such as nitrogen-fixing bacteria. Either one of

the two reasons (plant defenses or lack of plant virulence by bacteria) could explain why the

majority of the plant samples were unaffected by the inoculation of the bacterial isolates; against

known plant pathogens, the onion peels and lettuce leaves were effectively macerated. From the

plant models, it could be seen that the yellow onion and lettuce may have antibacterial

phytochemicals to aid their defense against the bacterial invasions. These phytochemicals could be a source for antibiotic development [30].

The fungal-bacterial interaction showed that the pathogenic fungus *A. fumigatus* was able to inhibit or outcompete approximately half of the isolates (most of which were Gram-positive bacteria) while *C. albicans* was able to inhibit a few of the isolates (most of which were Gram-negative bacteria). *A. fumigatus* has been found to secrete antibacterial compounds which would explain the ability to inhibit half of the isolates [41]. *C. albicans* may also be able to inhibit the bacteria with antibacterial compounds. These compounds could be extracted and developed into antibiotics that are selective in terms of Gram stain (*e.g.* antibacterial compounds from *A. fumigatus* could be developed into antibiotics targeting Gram-positive bacteria). Certain fungi have been known to contain antibacterial compounds that have been extracted and developed into commonly used antibiotics such as penicillin from the fungus *Penicillium* [16].

Many isolates were unable to utilize most antibiotics as a sole carbon source, suggesting that resistance did not result in the catabolism of the antibiotic. Though most of the isolates were able to consume colistin, the concentration for colistin was much lower than the others (*i.e.* 0.10 g/L instead of 0.50 g/L). Should this assay for colistin be repeated, colistin should be repeated with 0.50 g/L concentration. Out of the isolates that were able to utilize the antibiotics, the majority of them were isolated from soil environmental niches. This is expected since the majority of antibiotic-producing microbes (e.g. *Streptomyces* species) are commonly found in soils; thus bacteria naturally found in soils will need to have resistance mechanisms but they do not have to include antibiotic consumption/breakdown in their defense arsenal.

When examining the most common genera found in this study, the most prevalent groups (*i.e. Bacillus*, *Serratia*, and *Sphingobacterium*) appeared to be highly resistant to the antibiotics

colistin, kanamycin, penicillin, and trimethoprim, and they were capable of utilizing the antibiotics as a sole carbon source. In regards to the fungal-bacterial interaction, both *Bacillus* and *Serratia* are inhibited by *A. fumigatus* while *Sphingobacterium* was able to outcompete the fungus. In the case of *C. albicans*, only *Serratia* was inhibited. From this, though *Bacillus* and *Serratia* are insensitive to almost half of the antibiotics used, the fungi were able to inhibit the bacteria through secretion of antibacterial compounds. Since the most prevalent genera are found in all four environments (*i.e.* association with animals or fungi, plants, water, and soils), resistance could be spread easily to other neighboring bacteria. These specific antibiotic resistant bacteria can be suppressed (with the exception of *Sphingobacterium*) by *A. fumigatus*'s antibacterial compound, which could make *A. fumigatus* a possible source of antibiotic development.

The main hypotheses of this project were that (i) these bacterial isolates would be resistant to a large collection of antibiotics, (ii) the bacterial isolates collected from soil environments would be able to consume some of the nine antibiotics as a carbon source, (iii) some bacterial isolates would be plant pathogens, and (iv) some bacterial isolates would be inhibited by antibacterial compounds produced by the fungi. From the assays, all hypotheses were strongly supported with the exception of one. The hypothesis that was not strongly supported was that some isolates were plant pathogens. Though some isolates showed plant tissue maceration, the data were not substantial enough to warrant the assignation of a plant pathogen (i.e. for most of them, either 0 or 1 replicate out of 3 showed mild tissue destruction). Though only a small sample number was collected, it can be generalized to the greater population of native bacteria within Kentucky to a certain extent. These bacteria are resistant to an abundant of common antibiotics and may be less susceptible to others. This continuous rise of

increased resistance could spread to other non-resistant bacteria. With the discovery of plant models and fungal interaction capable of inhibiting the bacteria collected, sources of antibiotics could be discovered from these assays. Native bacteria within Kentucky have shown increasing resistance towards common antibiotics. Though microbial interaction (*e.g.* conjugation or fungal-bacterial interaction) may increase the spread of antibiotic resistance, reevaluation of antibiotic usage and development of new antibiotics from potential sources (*e.g.* plant models and fungi secretion) may allow the healthcare to be one step ahead in the arms race against bacteria.

Acknowledgements

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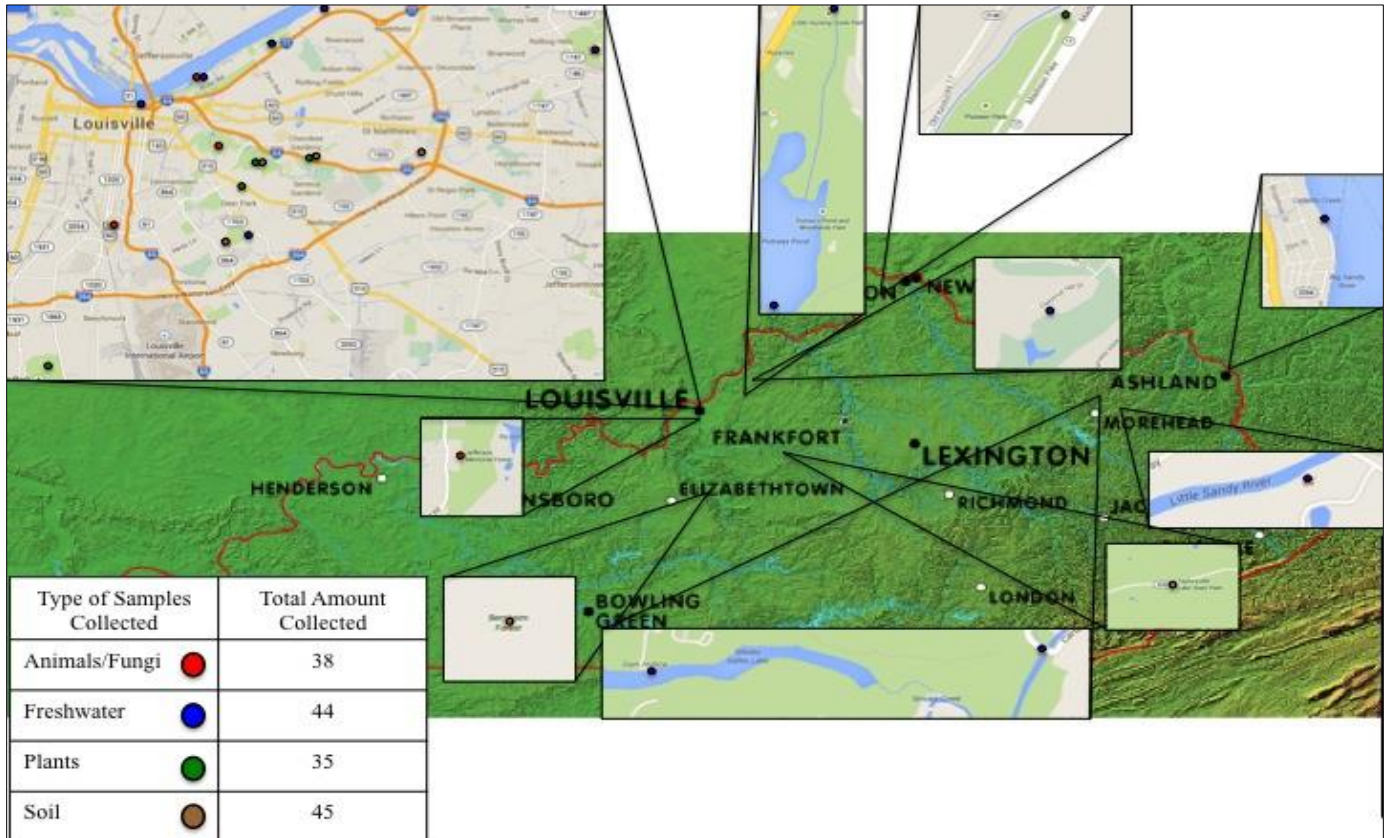
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Appendix

I. Supplemental Figures
II. Supplemental Tables

I. Supplementary Figures

Type of Samples Collected		Total Amount Collected
Animals/Fungi	🔴	38
Freshwater	🔵	44
Plants	🟢	35
Soil	🟤	45

Supplementary Figure 1. Sampling Locations in Kentucky A variety of samples collected from various sites in Kentucky in early July. Samples


Location Sampled	Amount Collected
Cherokee Park	14
Iroquois Park	1
Seneca Park	14
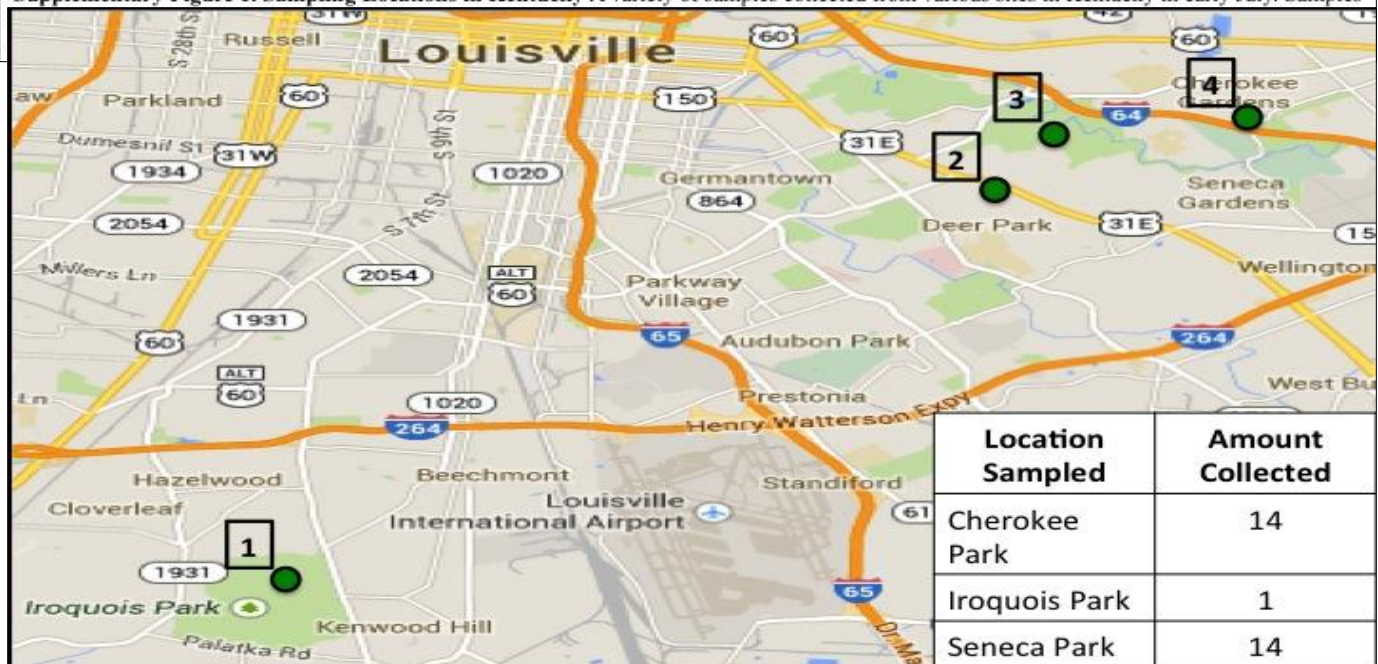
* The green dots represent sample sites

Supplementary Figure 2. Sampling Locations in Louisville. Samples associated with plant life were collected at distinct locations in Louisville in early June. Two of the samples were collected from vegetables bought at Kroger. Samples were collected at different times of the day, temperature, and heights relative to the ground.

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Supplementary Figure 3. Onion Infection Assay. Photos of the onion peels of a representative trial over a period of 2 days with photos taken in 24 hrs post infection. The positive control is circled in black. The negative control is shown in a red circle.
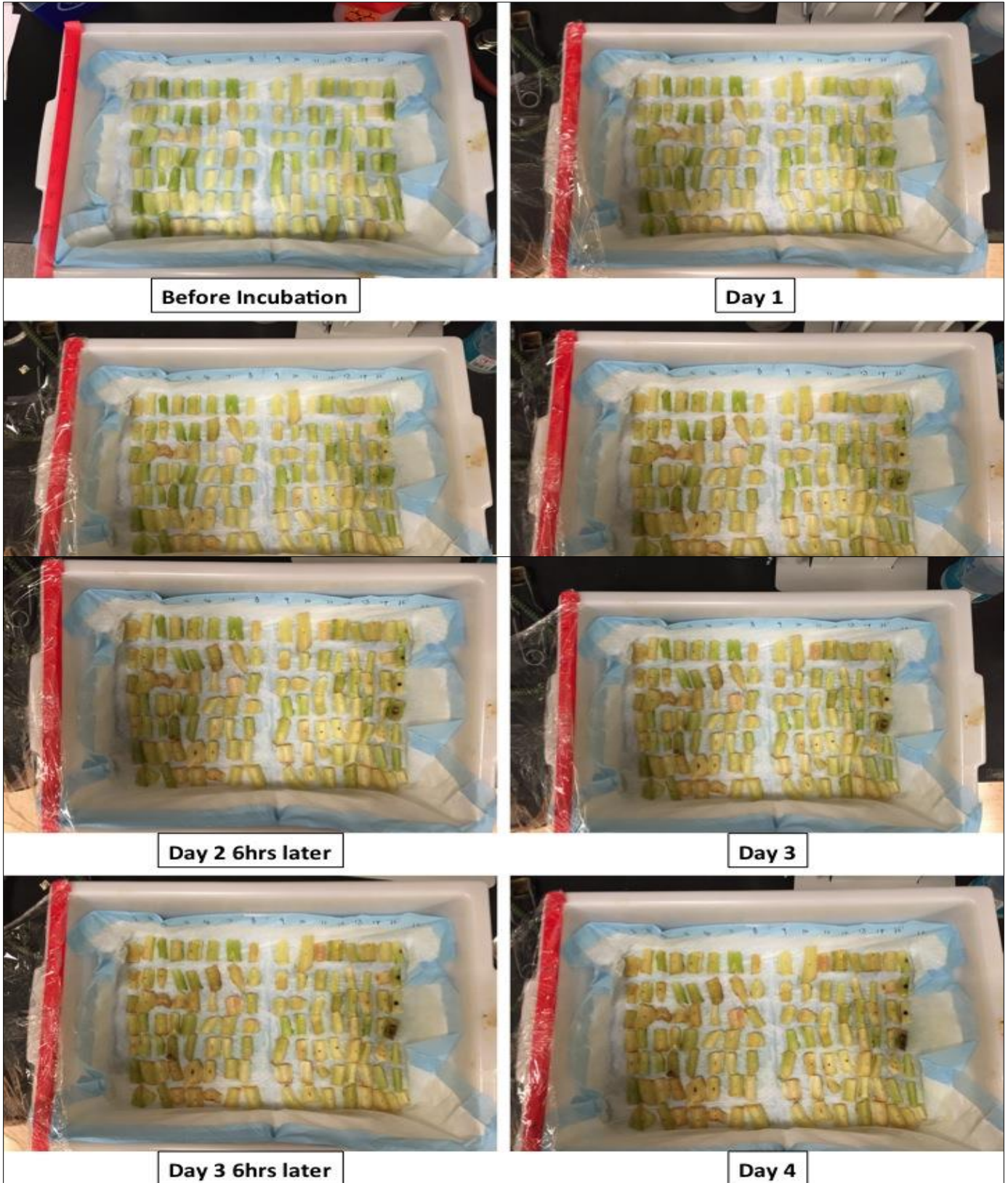
Supplementary Figure 4. Lettuce Infection Assay. Photos of the lettuce cores over a period of 5 days with photos taken in 6 hrs and 12 hrs post infection. Controls are the farthest right column of lettuce cores with the top four cores (black circles) as the positive controls and the bottom three (red circles) as the negative controls.

**Supplementary Figure 4** *continued*. **Lettuce Infection Assay**. Photos of the lettuce cores over a period of 5 days with photos taken in 6 hrs and 12 hrs post infection. Controls are the farthest right column of lettuce cores with the top four cores (black circles) as the positive controls and the bottom three (red circles) as the negative controls.

A.

B.

Bacterium growth unaffected by the fungus.

Bacterium growth affected by the fungus.
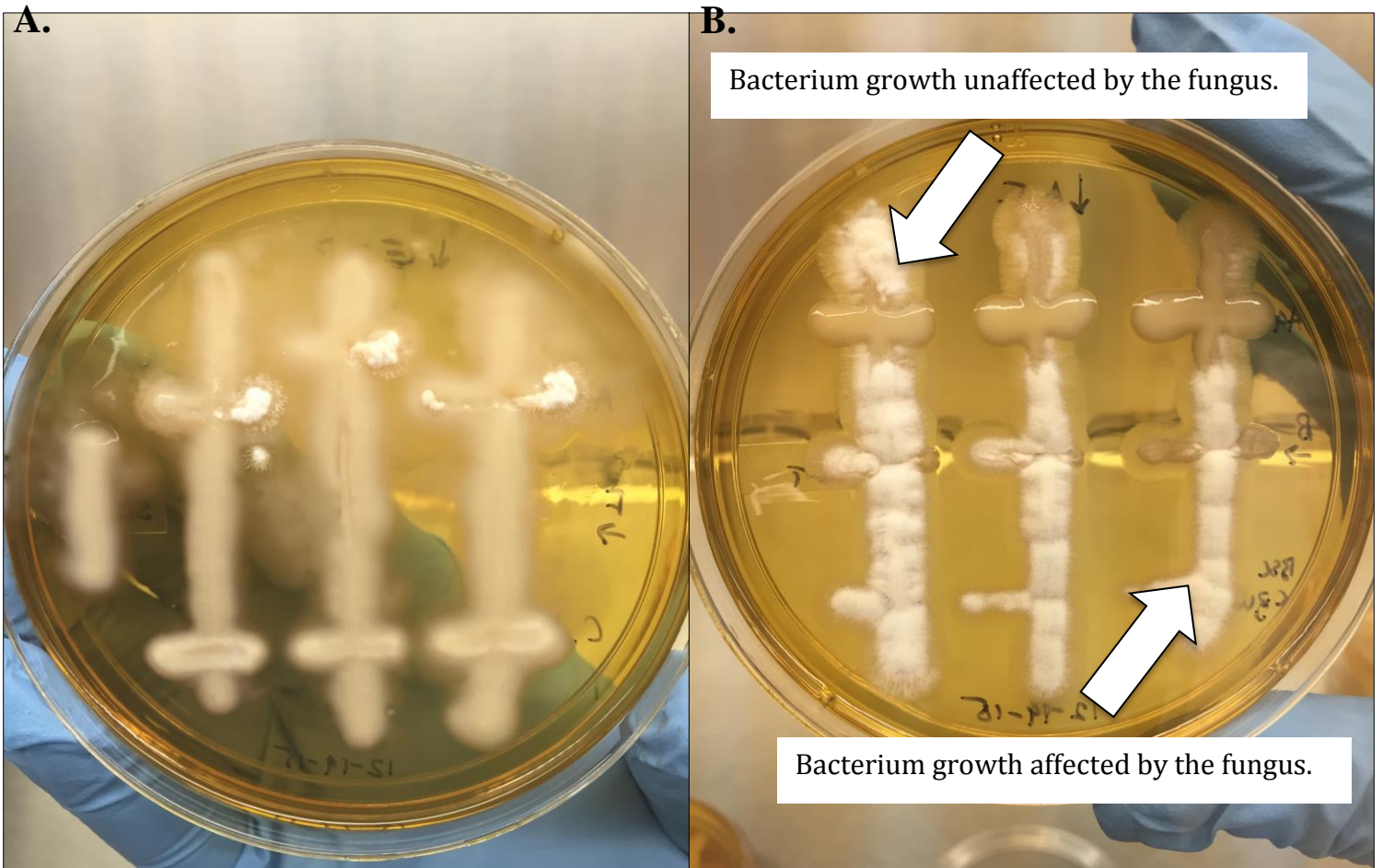
Supplementary Figure 5. Fungal-Bacterial Interaction Assay. Plates of two isolates to show the streaking pattern. In Panel A, the bacterial strain *Arthrobacter nitroguajacolicus* was streaked vertically followed by horizontal cross hatches of *A. fumigatus* (top three streaks) and *C. albicans* (bottom three streaks). In Panel B, *A. fumigatus* was struck vertically followed by streak of horizontal cross hatches of *Chryseobacterium stagni; Chryseobacterium gambrini* (top three streaks), *Pseudomonas protegens* (middle three streaks), and *Paenibacillus apiarius* (bottom three streaks).

II. Supplementary Tables

Supplementary Table 1. Species identification and abbreviations.

Bacterial Isolates	Abbreviations	MIC Abbreviations
1. *Aeromonas caviae*	*Aeromonas caviae*	*Aeromonas caviae*
2. *Aeromonas hydrophila*	*Aeromonas hydrophila*	*Aeromonas hydrophila*
3. *Aminobacterium*	*Aminobacterium*	*Aminobacterium*
4. *Arthrobacter nitroguajacolicus*	*Arthrobacter nitroguajacolicus*	*Arthrobacter nitroguajacolicus*
5. *Bacillus cereus*	*Bacillus cereus*	*Bacillus cereus*
6. *Bacillus thuringiensis; Bacillus anthracis; Bacillus cereus*	*Bacillus thuringiensis, -anthracis, -cereus*	*B. thur., -anthracis, -cereus*
7. *Bacillus thuringiensis; Bacillus cereus; Bacillus toyonensis*	*Bacillus thuringiensis, -cereus, -toyonensis*	*B. thur.,-cereus,-toyonensis*
8. *Brevibacillus laterosporus*	*Brevibacillus laterosporus*	*Brevibacillus laterosporus*
9. *Brevibacillus reuszeri; Brevibacillus choshinensis; Brevibacillus brevis*	*Brevibacillus reuszeri, -choshinensis, -brevis*	*B. reus.,-choshinensis,-brevis*
10. *Burkholderia cepacia*	*Burkholderia cepacia*	*Burkholderia cepacia*
11. *Cellulosimicrobium funkei; Cellulosimicrobium cellulans*	*Cellulosimicrobium funkei, -cellulans*	*C. funkei,-cellulans*
12. *Chromobacterium aquaticum*	*Chromobacterium aquaticum*	*Chromobacterium aquaticum*
13. *Chryseobacterium letacus*	*Chryseobacterium letacus*	*Chryseobacterium letacus*
14. *Chryseobacterium stagni; Chryseobacterium gambrini*	*Chryseobacterium stagni, -gambrini*	*C. stagni,-gambrini*
15. *Cloacibacterium sp./unknown*	*Cloacibacterium sp./unknown*	*Cloacibacterium sp./unknown*
16. *Elizabethkingia meningoseptica; Elizabethkingia anophelis*	*Elizabethkingia meningoseptica, -anophelis*	*E. meningoseptica,-anophelis*
17. *Empedobacter brevis*	*Empedobacter brevis*	*Empedobacter brevis*
18. *Enterococcus casseliflavus; Enterococcus gallinarum*	*Enterococcus casseliflavus, -gallinarum*	*E. casseliflavus,-gallinarum*
19. *Falsibacillus*	*Falsibacillus*	*Falsibacillus*
20. *Flavobacterium sp.*	*Flavobacterium sp.*	*Flavobacterium sp.*
21. *Leucobacter chromiireducens*	*Leucobacter chromiireducens*	*Leucobacter chromiireducens*
22. *Microbacterium arborescens*	*Microbacterium arborescens*	*Microbacterium arborescens*
23. *Morganella morganii*	*Morganella morganii*	*Morganella morganii*
24. *Myroides odoratus*	*Myroides odoratus*	*Myroides odoratus*
25. *Paenibacillus alvei*	*Paenibacillus alvei*	*Paenibacillus alvei*
26. *Paenibacillus apiarius*	*Paenibacillus apiarius*	*Paenibacillus apiarius*
27. *Proveidencia*	*Proveidencia*	*Proveidencia*
28. *Pseudochrobactrum asaccharolyticum; Pseudochrobactrum saccharolyticum*	*Pseudochrobactrum asaccharolyticum, -saccharolyticum*	*P. asac.,-saccharolyticum*
29. *Pseudomonas protegens*	*Pseudomonas protegens*	*Pseudomonas protegens*
30. *Rhodococcus equi*	*Rhodococcus equi*	*Rhodococcus equi*
31. *Serratia marcescens*	*Serratia marcescens*	*Serratia marcescens*
32. *Serratia marcescens*	*Serratia marcescens*	*Serratia marcescens*
33. *Serratia marcescens; Serratia nematodiphila*	*Serratia marcescens, -nematodiphila*	*S. marcescens,-nematodiphila*
34. *Sphingobacterium faecium*	*Sphingobacterium faecium*	*Sphingobacterium faecium*
35. *Sphingobacterium siyangensis; Sphingobacterium multivorum*	*Sphingobacterium siyangensis, -multivorum*	*S. siyangensis,-multivorum*

*All bacterial isolates used in this study with abbreviations to allow less space usage in some data tables and data figures.