Fungal-Bacterial Interactions Effects on Bacterial Antibiotic Resistance

Abstract

Antibiotic resistance by bacteria is a continued public health challenge due its impact on human health and wellbeing. While attributed to the overuse of antibiotics by humans, resistance has existed in nature prior to human introduction of antibiotics -- indicating that there are mechanisms in place that mediate the spread of resistance in bacteria and maintain it in the natural environment. It is hypothesized that interactions among microbes is one of these mechanisms. In the soil environment, fungal-bacterial interactions are ubiquitous, and their interactions are essential drivers of environmental processes. It is currently not known whether fungal-bacterial interactions is a mechanism that maintains bacterial antibiotic resistance in the soil. This project will test the hypothesis that interactions with fungi will help bacteria to overcome the effects of antibiotics, which will allow them to grow at higher concentrations. A bioassay of 35 bacterial strains with four fungal strains and four classes of antibiotics will be conducted in two phases: bacterial minimum inhibitory concentration (MIC) identification and bioassay of bacterial resistance in the presence of fungi via co-culture. Bacterial growth will be quantified via the Most Probable Number Method and a plate reader. The results from this experiment will contribute to understanding of how fungal-bacterial may contribute to antibiotic resistance and can guide future findings in antibiotic discovery and applications.

Key words: *Antibiotics, Antibiotic resistance, Fungal-bacterial interactions, Co-culture*

Introduction

Antibiotics are an essential part of human life – the development and advances of both human and animal medicine as well as of modern livestock production can be attributed to the discovery of antibiotics (7). Furthermore, antibiotics have extended human life expectancies by approximately 24 years from 1920 (3). Between the years 2000-2015, antibiotic consumption increased by 65%, and it is predicted that by 2030, global consumption will be higher by 200% (6). Despite the benefits, this excessive antibiotic consumption by humans has led to the selection of a plethora of antibiotic resistant microbes in the environment. The presence of antibiotics in the soil selects for antibiotic-resistant bacteria and can also facilitate the creation and transfer of antibiotic resistant genes (ARGs) (7). This transfer of ARGs can be detrimental if they are transferred to bacteria that can colonize the bodies of humans and animals.

Within the environment, specifically in the soil, antibiotics are to be a major driver of microbial communities. Antibiotic resistant bacteria can interact with other microbes in the soil, such as fungi. Fungi are particularly of interest, as they are well-known producers of secondary metabolites, such as antibiotics or antimicrobials (12). Fungi and bacteria coexist in all soil environments on the planet, therefore it can be expected that bacteria would have developed mechanisms to resist fungal antibiotics. This may explain the many observations of antibiotic resistance across many pristine soil environments with little human contact (24). However, whether fungal-bacterial interactions can change bacterial antibiotic resistance remains to be tested. Thus, the goal of this project is to test whether fungal-bacterial interactions can change the levels of antibiotic resistance in soil bacteria.

Significance: This project will aim to contribute novel findings to the mechanisms that maintain antibiotic resistance in the environment. Antibiotic resistance in microbes is immensely important to both health and the environment - as antibiotic resistance increases in the environment, the more dangers it will pose to human well-being and quality of life. Further, the findings of this project will serve as a foundation for future experiments that explore ways in which microbial interactions can contribute to antibiotic resistance and can help us develop ways to mitigate resistances of microbes that regularly come into contact with humans..

Hypothesis: Direct interactions of fungi with bacteria allow bacteria to grow at higher concentrations of antibiotics (higher resistance). These interactions will also allow bacteria to grow in more classes of antibiotics.

Literature Review

Antibiotics and Resistance on Human Health

Antibiotics, medicines that are isolated primarily from microbes and are used to fight and manage bacterial infections, have significant impacts on human health, animal health, and the environment (5). While antimicrobials have been used extensively before modern medicine, the 1928 discovery of the first modern antibiotic (Penicillin) is attributed to Alexander Fleming (3). Antibiotic discovery and introduction then spiked, peaking in the 1950s (2).

Antibiotics are broadly classified based on which category of bacterial cell mechanism they target - cell wall synthesis, nucleic acid synthesis, and protein synthesis. Within these areas, there are specific subsets of targets, including but not limited to: beta-lactam agents, RNA polymerase, and the 50S subunit of the bacterial ribosome. Each type of antibiotic works differently, but ultimately the antibiotics will often disrupt and/or inhibit a particular process to kill off the bacterium, or prevent it from replicating further (1). While antibiotics were made with the intent to exterminate bacteria, it has unintentionally made bacteria more resistant to these antibiotics.

Unfortunately, modern day antibiotic usage is also coupled with antibiotic resistance - meaning that the bacteria causing the infection is resistant to the regular doses of antibiotics, since the bacteria contain genes that confer resistance (8). A major cause of this resistance can be attributed to the pressures exerted by excessive human antibiotic use, both on humans and on animals (6). The more frequently antibiotics are consumed by human and animal bodies, the more resistant bacteria emerge (3). In addition, this usage can lead to excessive amounts of antibiotic deposit in the environment. For instance, approximately 75-80% of tetracyclines (broad-spectrum) and 50-90% of erythromycin (broad-spectrum) is discharged via feces or urine

(7). Since humans and animals are unable to completely metabolize antibiotics, antibiotic resistance escalates.

The World Health Organization (2019) has listed 5 classes of antibiotics as the highest priority critically important antimicrobials (10). These 5 classes are characterized such that they are essential -- that is, they are the only or one of very few treatments available -- in human medicine and thus require urgent risk management for antibiotic resistance (10). To make matters worse, there has been a decline in antibiotic discovery, due to lack of economic return in the field, regulatory barriers, and fear of worsening the current resistance situation (3, 9). These two issues -- rising resistance and less discovery -- have considerable effects on the medical landscape. Resistance is now becoming more common among pathogens like TB causing *Mycobacterium tuberculosis* and pneumonia causing *Pseudomonas aeruginosa*, and continues to be a problem in pathogens such as Methicillin-resistant *Staphylococcus aureus* (MRSA) (3). These infections require more antibiotics and often a longer hospital stay by approximately 6.4-12.7 days, which can burden families and the healthcare system with additional costs.

Dissemination of Resistance via Antibiotic Resistance Genes (ARGs)

It is very important to note that antibiotic resistance has been prevalent prior to the start of human antibiotic interaction with the environment (4, 8). Therefore, resistance cannot be a consequence of human use alone, and that underlying mechanisms have already developed in nature. In fact, several functions of antibiotic production and resistance in bacteria have been postulated: to maximize survivability in competitive interactions between other microorganisms, to function as signal molecules/to communicate and to inform the host's immune response (4).

A combination of mechanisms, forces, and selective pressures (including human-based) mediate the spread of bacteria and their antibiotic resistance genes (ARGs) (8). Bacteria are able to undergo horizontal gene transfer (HGT), the transfer of DNA and thereby genes, via three different mechanisms: conjugation, transduction, and transformation (11). In conjugation, bacteria directly transfer their genetic material via pilus to another bacteria; in transduction, bacteriophages introduce genetic material; and in transformation, bacteria take up genetic information from the environment (11). In this way, once a singular bacterium contains the antibiotic resistance gene, it is able to spread it throughout an entire population and additionally replicate itself and its genes. Physical and biological forces, such as wind or animals also contribute to the spread of ARGs since both are able to carry microbes far distances (8). Specifically for animals, it has been found that the closer their proximity to humans (such as pets, or those in densely populated areas) are prime reservoirs for ARGs [REF].

The Role of Fungi

Within the environment, there exist another vital set of microorganisms - fungi. Fungi are particularly important for the soil ecosystem, as they act primarily as decomposers and therefore act as regulators of nutrient and matter cycling (13). Their presence also helps to maintain soil health. Fungi, like bacteria, are also large producers of secondary metabolites, molecules that are not required for growth but are essential for survival, such as antibiotics (14). At this time, around 40% of filamentous fungi are thought to produce antibiotics (12). In addition, some filamentous fungi have been studied due to their potential to degrade antibiotics (15). In particular, the *Mucoromycotina* fungi and *Ascomycota* fungi are of attraction for its potential in

degrading fluoroquinolones. Thus, fungi are of particular interest in the antibiotic and drug production fields.

Fungal-Bacterial Interactions on Antibiotic Resistance

Fungal-bacterial interactions in environments, such as soil systems, prove to be a major driver of environmental processes, since fungi and bacteria have the ability to associate with each other through physical and/or chemical interactions (16). Interestingly bacteria, like fungi, can also contain genes that degrade or inactive antibiotics (7). For this reason, the most studied fungal-bacterial interaction is antibiosis or the dispersal of damaging molecules to the other party (16). In other words, the fungi and bacteria are thought to be undergoing an "evolutionary arms race", where fungi and bacteria are constantly adapting mechanisms to combat each other's biological warfare through production or degradation of antibiotics (17). On the flip side, fungi and bacteria can hold beneficial relationships including one of two forms of a symbiotic relationship - ectosymbiotic or endosymbiotic (16). For instance, in biofilm structures, the presence of the fungus *Candida albicans* can enhance the antibiotic resistance capacity of the bacteria *Staphylococcus aureus*. Furthermore, soil fungi are able to contribute to horizontal gene transfer mechanisms and help to spread ARGs (20).

Due to these intricate interactions and necessary communications between fungi and bacteria, it can be difficult to understand the molecular mechanisms *in-vitro* (18). Since these microorganisms are often in contact with one another, they may not elicit their usual responses if they are isolated separately. As a result, the co-culturing method, the growing of both fungi and bacteria together in the same chamber, can be used to overcome this limitation since it facilitates

a similar environment in nature. One study utilized this fungal-bacterial co-culturing methodology and was successful in producing significant amounts of antibiotics (19).

Methodology/Research Design

Selection of antibiotics, fungal and bacterial strains

From the Nguyen Lab antibiotics arsenal, 4 of the "Highest Priority Critically Important" antibiotics (Cefepime, Vancomycin, Erythromycin, Levofloxacin) were chosen as determined from the *Critically Important Antimicrobials for Human Medicine (6th rev.)* (10). The World Health Organization (WHO) has designated five classes out of 35 antimicrobials as the highest priority and critically important based on various criteria and prioritization factors: Quinolones, newer generation Cephalosporins, Macrolides/Ketolides, Glycopeptides, and Polymyxins.

Furthermore, 35 bacterial strains were selected from 865 total strains isolated from Waimea Valley using the Nguyen Lab's culture collection. Specifically, four genera were chosen -- *Chryseobacterium*, *Bacillus*, *Stenotrophomonas*, and *Rhodococcus*-- based on their various levels of resistances and importance to human health.

Three fungal strains from another Nguyen Lab culture collection will be selected. The fungal strains belong to several groups of fungi, including the Phyla Basidiomycota, Ascomycota, and Mucoromycota with ranging potentials to produce antibiotics in culture. From each phylum, two cultures will be selected and grown in liquid media (Modified Melin-Norkrans). The three fungal strains will then be chosen based on their growth characteristics (slow, medium, or fast).

Phase 1: Identification of bacterial minimum inhibitory concentration (MIC)

The first phase of the project is to determine each bacterial isolates' minimum inhibitory concentration (MIC) using a standardized curve on a plate reader. Determination of the MIC in Phase 1 is critical for Phase 2, since it will be used as a baseline of resistance.

Bioassay plate preparations

The bioassays will be performed in 48 well cell-culture plates. For each plate with Mueller Hinton broth as the medium, 4 different antibiotics will be added in successive rows with increasing concentrations down the plate (Fig 1). The first well of each vertical row will contain no antibiotics to act as a control to determine if the bacteria is properly growing. There will be a total of 36 plates (one plate per two bacterial isolates; two biological replicates). Antibiotic stock solutions will be prepared based on the Nguyen Lab Antibiotics for Culture Media Protocol. The various required concentrations of the antibiotics will be prepared via either a dilution of the stock solution or via the making of a further concentrated solution.

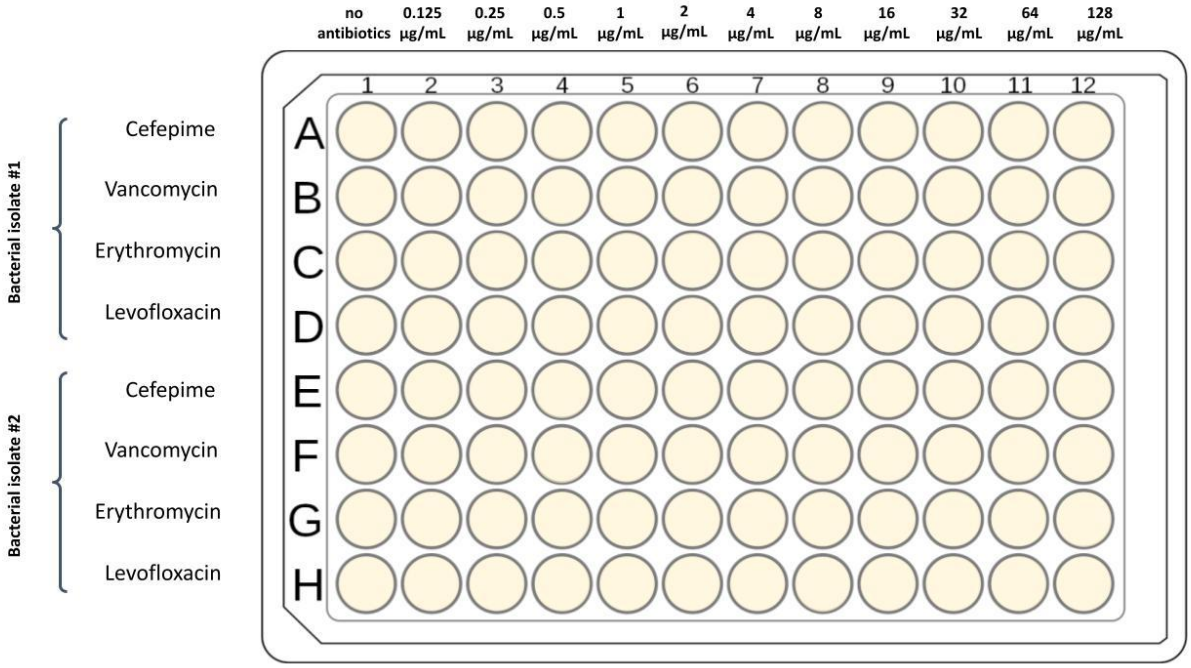

Figure 1. A schematic representation of the 48-well culture plate to be used in this experiment. Two bacterial isolates will be added per plate. Concentrations of the antibiotics will range from 0 μg/mL to 128 μg/mL.

Culture preparations

First, actively growing bacteria will be suspended in a tube containing 5 ml of phosphate buffer saline (PBS) . The concentration of cells will be determined via a spectrophotometer at OD600 (absorbance spectra at 600 nm). All bacterial isolates will be diluted so that they are inoculated into the culture using a single value of the absorbance spectra – in other words, the same number of cells will be inoculated.

Bioassay plate inoculation

A standardized number of cells will be inoculated into each well of the prepared bioassay plate. The plates will be incubated at 35-37 °C for 18-20 hours (22) and will be loaded into the plate reader, where the growth curves will be determined. The lowest concentration of antibiotic that prevents bacterial growth will be chosen as the MIC for that bacteria and specific antibiotic.

Phase 2: Bioassay of bacterial resistance in the presence of fungi

The second phase is to bioassay resistance in bacteria when grown with fungi. All bacteria cultures will be grown either on their own or with a fungus in factorial combinations and controls: 1. Baseline Control (bacteria + antibiotics to measure the standard growth with no fungal interactions), 2. Positive Control (fungi + bacteria + no antibiotics to measure growth with only fungal interactions), and 3. Treatment (Fungi + bacteria + antibiotics to measure growth with fungi and the presence of antibiotics). The fungal isolates will be pre-cultured for four days

followed by bacteria. All bioassays will be performed in 48-well microtiter plates in Mueller-Hinton broth, and incubated at 37 °C for 1 day (23). This experiment will be replicated four times, and may be further replicated to achieve stronger data.

Bacterial growth measurement

Bacterial growth of Phase 2 will be measured through serial dilutions, plating, and total colony forming units estimated by the most probable number method. A second set of measurements using qPCR may be used to validate these results. All data will be recorded and organized on a Google Spreadsheet (separate from the MIC spreadsheet).

Data analysis

Data will be statistically compared using multivariate analysis of variance (MANOVA) in R, as there are multiple variables that will be measured in this project. The influence of the three groups (baseline control, positive control, treatment) on antibiotic resistance will be explored by plotting against the MIC and bacterial growth measurements.

Lab Trainings Completed

The student has completed all requirements to conduct this project in the Nguyen Laboratory: RCR Training (Biomedical and Biological; CITI), ORC101 (General Biosafety), and UHM EHSO Lab Safety Checklist. The student will take the UHM EHSO Lab Safety Checklist refresher in October 2022 and the ORC101 refresher course in January 2023.

Role of the Researcher

The student will identify bacterial MICs, perform co-culture bioassays, and measure bacterial growth in this project. The student will perform and collect all data required. Further, the student will analyze the data collected from the above mentioned.

Timetable

Time (Month, Year)	**Goals**
August 2022	Start constructing and testing protocols.
September 2022	Begin waking up required bacterial and fungal isolates and make sure they are all viable. Perform any preliminary growth experiments as necessary.
October 2022	Begin Phase 1. Also analyze the data from Phase 1 to determine the MICs for each isolate. Take the UHM EHSO Lab Safety Refresher.
November - December 2022	Begin Phase 2. Start to outline Senior Honors Thesis.
January 2023	Initial analysis of bioassay data. Repeat experiments here as needed. Finish drafting all sections except for the results/discussion. Take ORC101 refresher.
February 2023	Learn to work with ANOVA and R and start data analysis. First full draft of thesis.
March 2023	Complete analysis of all collected data. Turn in the final draft of the thesis to mentor and committee member(s) for review.
April 2023	Undergraduate showcase. Submit final Senior Honors Thesis.
May 2023	Graduation.

Resources and Material Available to the Researcher

All resources and materials will be available to the student through the Nguyen Lab at the University of Hawai‘i at Mānoa.

Conclusion

As research on antibiotic resistance becomes increasingly imperative, so does the importance of understanding the different facets for the development and spread of resistance. The relative lack of information on both antibiotic resistance and fungal-bacterial interactions presents a barrier to the growth of the field. The findings of this study are expected to show that bacteria co-cultured with fungi develop a stronger resistance than those cultured alone, suggesting that these interactions play a vital role in antibiotic resistance mechanisms in nature. This foundational research will allow for future studies in fungal-bacterial interactions and contribute further knowledge in mitigating this public health crisis.

Citations

1. Kapoor, G., Saigal, S., & Elongavan, A. (2017). Action and resistance mechanisms of antibiotics: A guide for clinicians. Journal of Anaesthesiology, Clinical Pharmacology, 33(3), 300–305. https://doi.org/10.4103/joacp.JOACP_349_15
2. Hutchings, M. I., Truman, A. W., & Wilkinson, B. (2019). Antibiotics: Past, present and future. Current Opinion in Microbiology, 51, 72–80. https://doi.org/10.1016/j.mib.2019.10.008
3. Ventola, C. L. (2015). The antibiotic resistance crisis. Pharmacy and Therapeutics, 40(4), 277–283. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4378521/
4. Sengupta, S., Chattopadhyay, M. K., & Grossart, H.-P. (2013). The multifaceted roles of antibiotics and antibiotic resistance in nature. Frontiers in Microbiology, 4, 47. https://doi.org/10.3389/fmicb.2013.00047
5. Clardy, J., Fischbach, M., & Currie, C. (2009). The natural history of antibiotics. Current biology : CB, 19(11), R437–R441. https://doi.org/10.1016/j.cub.2009.04.001
6. Klein, E. Y., Van Boeckel, T. P., Martinez, E. M., Pant, S., Gandra, S., Levin, S. A., Goossens, H., & Laxminarayan, R. (2018). Global increase and geographic convergence in antibiotic consumption between 2000 and 2015. Proceedings of the National Academy of Sciences, 115(15). https://doi.org/10.1073/pnas.1717295115
7. Cycoń, M., Mrozik, A., & Piotrowska-Seget, Z. (2019). Antibiotics in the soil environment—Degradation and their impact on microbial activity and diversity. Frontiers in Microbiology, 10. https://www.frontiersin.org/article/10.3389/fmicb.2019.00338
8. Allen, H. K., Donato, J., Wang, H. H., Cloud-Hansen, K. A., Davies, J., & Handelsman, J. (2010). Call of the wild: Antibiotic resistance genes in natural environments. Nature Reviews Microbiology, 8(4), 251–259. https://doi.org/10.1038/nrmicro2312
9. Plackett, B. (2020). Why big pharma has abandoned antibiotics. Nature, 586(7830), S50–S52. https://doi.org/10.1038/d41586-020-02884-3
10. World Health Organization. (2019). Critically important antimicrobials for human medicine (6th rev.). World Health Organization. https://apps.who.int/iris/handle/10665/312266
11. Burmeister, A. R. (2015). Horizontal gene transfer. Evolution, Medicine, and Public Health, 2015(1), 193–194. https://doi.org/10.1093/emph/eov018
12. Hautbergue, T., Jamin, E. L., Debrauwer, L., Puel, O., & Oswald, I. P. (2018). From genomics to metabolomics, moving toward an integrated strategy for the discovery of fungal secondary metabolites. Natural Product Reports, 35(2), 147–173. https://doi.org/10.1039/C7NP00032D
13. Frąc, M., Hannula, S. E., Bełka, M., & Jędryczka, M. (2018). Fungal biodiversity and their role in soil health. Frontiers in Microbiology, 9. https://www.frontiersin.org/article/10.3389/fmicb.2018.00707
14. Sanchez, S., & Demain, A. L. (2011). Secondary metabolites. Comprehensive Biotechnology (pp. 155–167). Elsevier. https://doi.org/10.1016/B978-0-08-088504-9.00018-0
15. Olicón-Hernández, D. R., González-López, J., & Aranda, E. (2017). Overview on the biochemical potential of filamentous fungi to degrade pharmaceutical compounds. Frontiers in Microbiology, 8. https://www.frontiersin.org/article/10.3389/fmicb.2017.01792
16. Frey-Klett, P., Burlinson, P., Deveau, A., Barret, M., Tarkka, M., & Sarniguet, A. (2011). Bacterial-fungal interactions: Hyphens between agricultural, clinical, environmental, and food

microbiologists. Microbiology and Molecular Biology Reviews, 75(4), 583–609. https://doi.org/10.1128/MMBR.00020-11

17. Boer, W. de, Folman, L. B., Summerbell, R. C., & Boddy, L. (2005). Living in a fungal world: Impact of fungi on soil bacterial niche development. FEMS Microbiology Reviews, 29(4), 795–811. https://doi.org/10.1016/j.femsre.2004.11.005
18. Vinale, F., Nicoletti, R., Borrelli, F., Mangoni, A., Parisi, O. A., Marra, R., Lombardi, N., Lacatena, F., Grauso, L., Finizio, S., Lorito, M., & Woo, S. L. (2017). Co-culture of plant beneficial microbes as source of bioactive metabolites. Scientific Reports, 7(1), 14330. https://doi.org/10.1038/s41598-017-14569-5
19. Ngwoke, K. G., Nwoye, V. O., Okoye, F. B. C., & Proksch, P. (2017). Staphylococcus aureus, Bacillus subtilis and Escherichia coli induced copious production of antibiotics in an overnight co-culture with three soil fungi. Journal of Current Biomedical Research, 1(1), 65–72. https://journals.unizik.edu.ng/index.php/jcbr/article/view/692
20. Nazir, R., Shen, J.-P., Wang, J.-T., Hu, H.-W., & He, J.-Z. (2017). Fungal networks serve as novel ecological routes for enrichment and dissemination of antibiotic resistance genes as exhibited by microcosm experiments. Scientific Reports, 7(1), 15457. https://doi.org/10.1038/s41598-017-15660-7
21. Grada, A., & Bunick, C. G. (2021). Spectrum of antibiotic activity and its relevance to the microbiome. JAMA Network Open, 4(4), e215357. https://doi.org/10.1001/jamanetworkopen.2021.5357
22. Andrews, J. M. (2001). Determination of minimum inhibitory concentrations. Journal of Antimicrobial Chemotherapy, 48(suppl_1), 5–16. https://doi.org/10.1093/jac/48.suppl_1.5
23. Schroeckh, V., Scherlach, K., Nützmann, H. W., Shelest, E., Schmidt-Heck, W., Schuemann, J., Martin, K., Hertweck, C., & Brakhage, A. A. (2009). Intimate bacterial-fungal interaction triggers biosynthesis of archetypal polyketides in Aspergillus nidulans. Proceedings of the National Academy of Sciences of the United States of America, 106(34), 14558–14563. https://doi.org/10.1073/pnas.0901870106
24. Van Goethem, M. W., Pierneef, R., Bezuidt, O. K. I., Van De Peer, Y., Cowan, D. A., & Makhalanyane, T. P. (2018). A reservoir of 'historical' antibiotic resistance genes in remote pristine Antarctic soils. Microbiome, 6(1), 40. https://doi.org/10.1186/s40168-018-0424-5