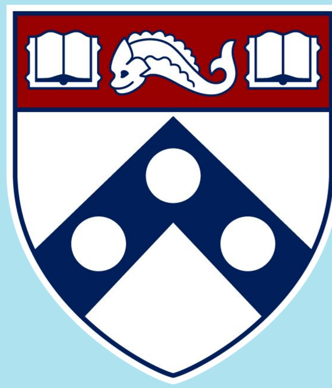

Investigating *Clathria prolifera* Holobiont and Metabolites

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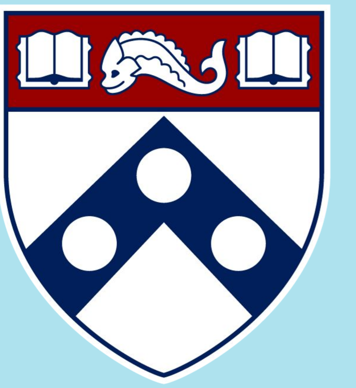


Background

Natural Products of C. prolifera

C. prolifera harbors a rich microbial community- which is characteristic of the class Demospongiae. The microbial inhabitants can produce compounds the sponge can not produce *de novo*; including antimicrobial and antiproliferative agents.

These products which are not required for metabolism are secondary metabolites, also known as natural products-. Once isolated, these natural products can be screened for bioactivity through assays and other methods.

Figure 1.
C. prolifera specimen before processing and utilization in the laboratory

Indraningrat, A.A.G.; Smidt, H.; Sipkema, D. Bioprospecting Sponge-Associated Microbes for Antimicrobial Compounds. *Mar. Drugs* **2016**, *14*, 87. https://doi.org/10.3390/md14050087

Carotenoids

Carotenoids are are hydrocarbon pigments ranging from red to yellow. They cannot be synthesized *de novo* by sponges, or humans; however, carotenoids can act as oxygen radical scavengers and UV protectants. Production is directly correlatated with exposure to UV as indicated by the increase in carotenoid concentration within *C. prolifera* during summer months.

Additionally, carotenoids are the precursor of retinol (vitamin A). Humans rely solely on consumption of carotenoids for retinol as they are unable to be produced *de novo*.

Research suggests that the carotenoid lycopene, and potentially other carotenoids, are anti proliferative and can be used as therapeutics to help treat cancer.

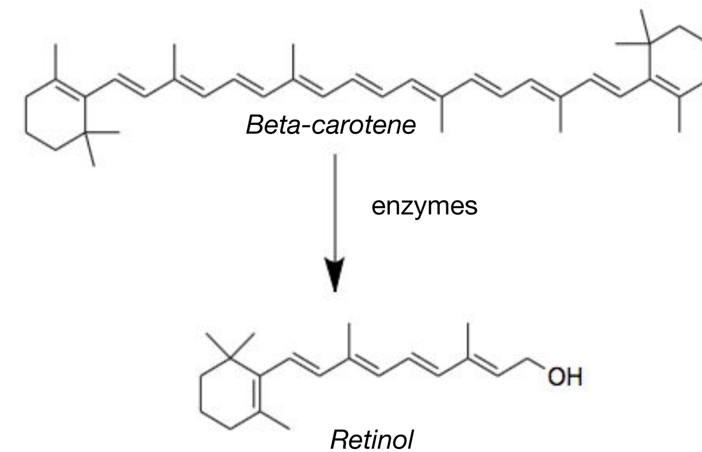
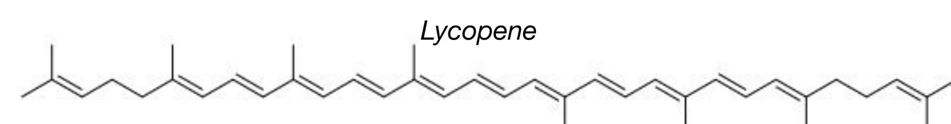


Figure 3.
(Above) Summary of the synthesis of retinal using Beta-carotene as a precursor

Figure 2.
(Below) The structure of lycopene



Hill, M. Plasticity of Acquired Secondary Metabolites in Clathria Prolifera (Demospongia: Poecilosclerida): Putative Photoprotective Role of Carotenoids in a TEMPERATE Intertidal Sponge. *The Open Marine Biology Journal* **2010**, *4* (1), 87–95.

Jang, H.-J.; Yoon, S.-H.; Ryu, H.-K.; Kim, J.-H.; Wang, C.-L.; Kim, J.-Y.; Oh, D.-K.; Kim, S.-W. Retinoid Production USING Metabolically ENGINEERED Escherichia Coli with a Two-Phase Culture System. *Microbial Cell Factories* **2011**, *10* (1), 59

Imran, M.; Ghorat, F.; Ul-Haq, I.; Ur-Rehman, H.; Aslam, F.; Heydari, M.; Shariati, M. A.; Okuskhanova, E.; Yessimbekov, Z.; Thiruvengadam, M.; Hashempur, M. H.; Rebezov, M. Lycopene as a Natural Antioxidant Used to Prevent Human Health Disorders. *Antioxidants* **2020**, *9* (8), 706.

Microbial 'Fight Club'

In order to stimulate microbial interactions, several various species of microorganisms- all isolated from *C. prolifera* and classified using 16S sequencing- were struck on a petri dish purposefully intersecting.

The co-cultivation of microbes gives visual data on the species specific microbial interactions as well as a hint at the secondary metabolic profile of each organism. By examining ecological effects, microbial colonies of interest are able to be selected for further investigation.

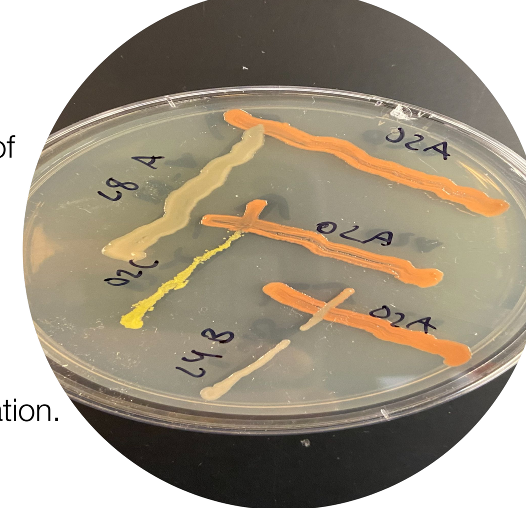


Figure 3.
Bacillus marisflavi(orange) struck against several other isolates to screen for bioactive substances. In this screening nothing of interest was found.

Isolation of Carotenoids

Methods

A small portion of sponge tissue was macerated using a mortar and pestle with acetone. Equal parts of celite were added. The solution was filtered with a Büchner funnel and filter paper. This process was repeated three times before the acetone was evaporated off using a rotovap. The extract was stored at -20°C to prevent degradation.

Column Chromatography

The extract was dissolved in acetone and partitioned with hexanes- removing the aqueous layer. Column chromatography was initially used to separate the solution into five fractions. The column was run with hexanes and ethyl acetate starting at 100% hexane and progressing in increments of 5% to 100% ethyl acetate.

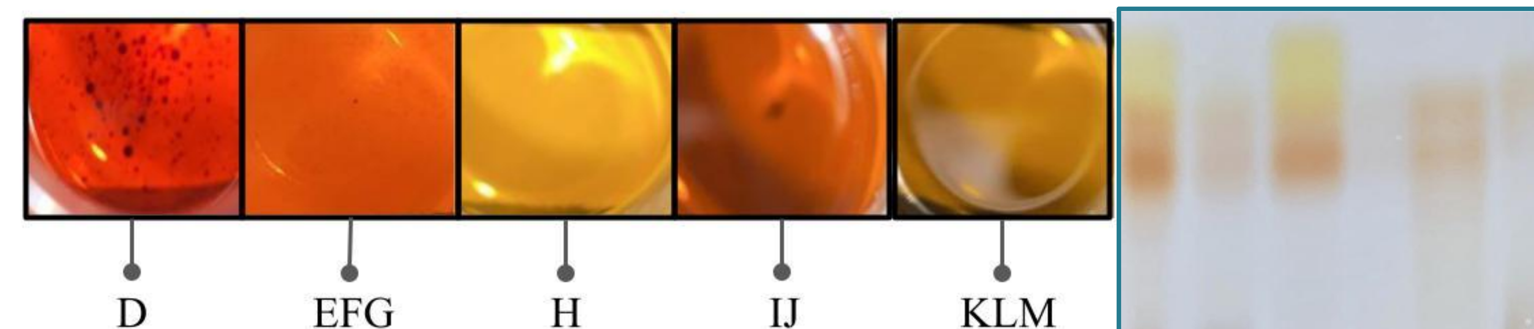


Figure 4.
The figure on the left displays the five fractions retrieved from the column. Each fraction was labelled alphabetically starting at A. Fractions with similar compounds- determined by analytical TLC were combined and relabelled as a combination of the two fractions previous names ordered alphabetically. For instance a combination of fraction E, F, and G resulted in fraction EFG. The figure on the rights shows a TLC plate run in a 95% hexane and 5% ethyl acetate solution with solutions- labelled from left to right- of the crude extract and fractions D, EFG, H, IJ, and KLM.

UV-Vis Spectroscopy Analysis

The characteristic orangish-red color of carotenoids allows for rough UV-Vis analysis. If the sample contained carotenoids they would display the evident peaks in the 400-500 nm region.

Each fraction was evaporated and the mass was recorded. The fractions were then redissolved in reagent alcohol to create a 0.001 g/ml solution. 800 ul of each fraction was added to a cuvette.

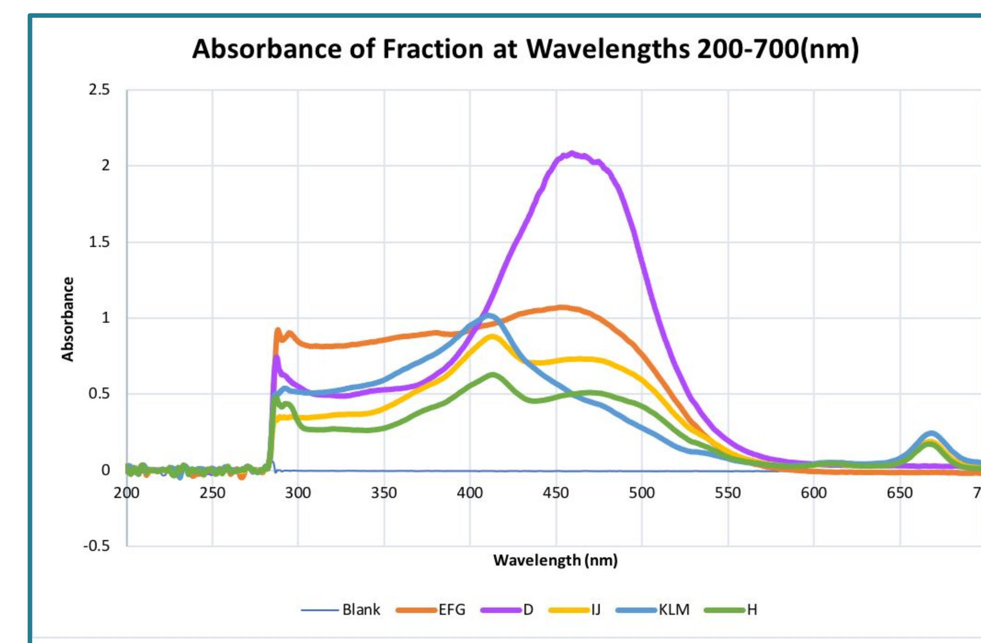


Figure 5.
Absorbance of fractions (pictures above) measured at wavelengths 200-700 nm. Measurements taken on a Thermo Fisher and DeNovix UV-Vis spectrophotometer

Based on the UV-Vis spectrophotometer measurements, there are several peaks on the graphs within 400-500 nm and it can be presumed that there are carotenoids within the solution. Additionally, due to the several peaks in each fractions it is assumed that there are many different compounds in each fraction.

Scott, K. J. Detection and Measurement of Carotenoids by Uv / Vis Spectrophotometry. *Current Protocols in Food Analytical Chemistry* **2001**, *00* (1).

Biological Assays of Fractions

Methods

Filter paper was cut into 8mm diameter circles using a hole punch. A 3x3 grid of filter papers was placed on a petri dish filled with marine broth agar. This was done to 3 petri dishes. 10ul 20ul and 50ul of fractions D, EFG, and H were spotted on the filter paper. This was done to two of the rows in the petri dish. The last row was spotted with just reagent alcohol as a control.

50 mL of marine broth agar was prepared and left in a water bath to cool. 5 mL of *Psychrobacter sp.* liquid culture was added and mixed into the agar. 15 mL of marine broth agar was added on top of each petri dish to create a filter paper assay.

Zhao, J. Z.; Grafius, E. Assessment of DIFFERENT BIOASSAY Techniques for Resistance Monitoring in the Diamondback MOTH (Lepidoptera: Plutellidae). *Journal of Economic Entomology* **1993**, *86* (4), 995–1000.

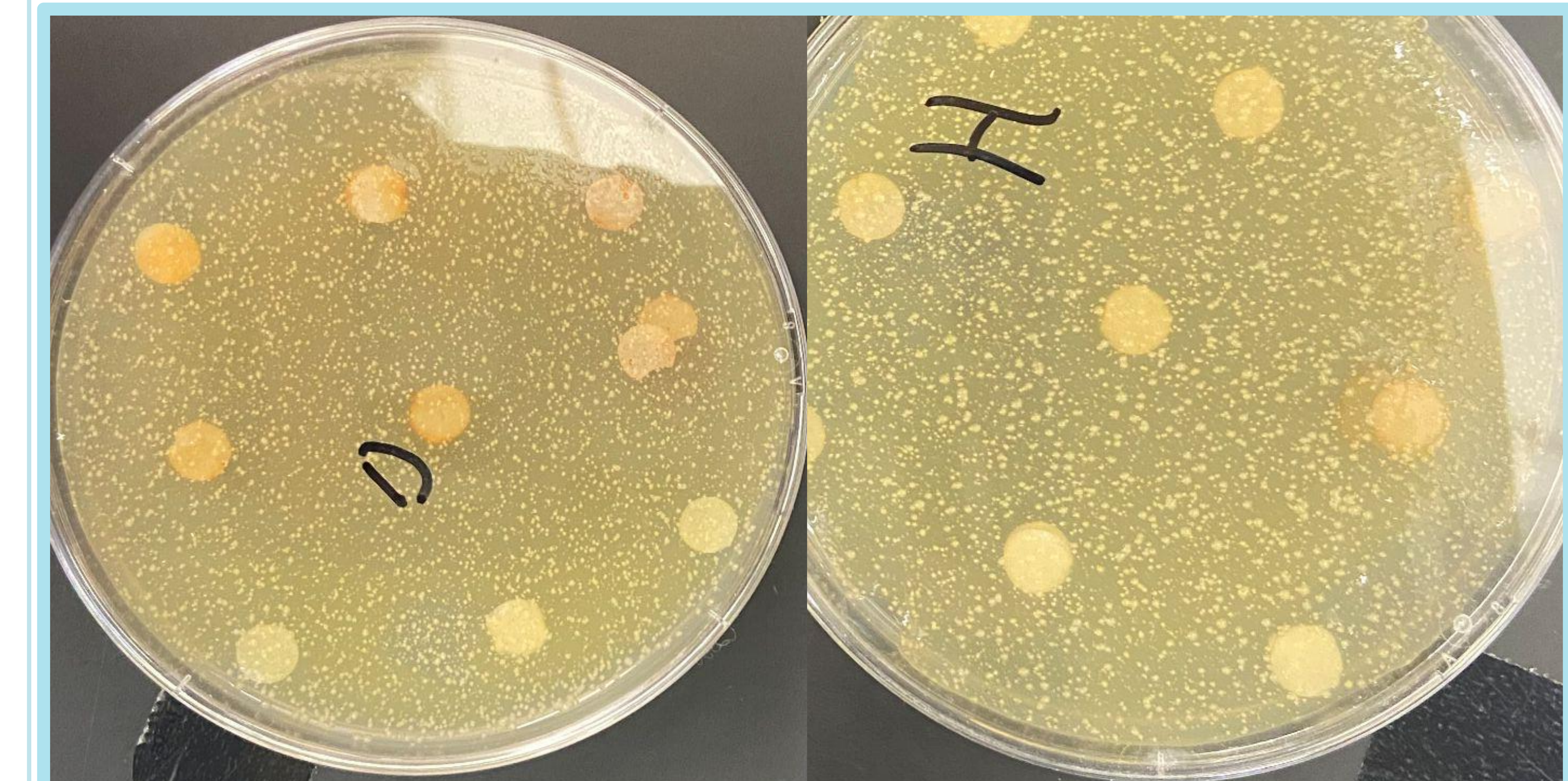


Figure 6.
Assays 2 days after pouring. Yellow colonies can be seen dotted throughout. In the picture above, the top 2 rows are from the columns labelled on the petri dish- D and H respectively. The leftmost rows had 10 ul added, the middle row had 20ul added, and the rightmost row had 50 ul added. Little to no biological activity was able to be observed from the from the assay.

Bacillus pumilus Antimicrobial Activity

16S Sequence Phylogeny

Each microbial isolate was submitted for 16S sequencing and submitted to BLAST NCBI for tentative phylogenetic identification. Pictured left are several isolates that were sequenced and classified.

Once each isolate received a tentative classification, they were struck together on petri dishes to attempt to force an interaction.

Interaction between B. pumilus and B. marisflavi

Each microbial isolate was submitted for 16S sequencing and submitted to BLAST NCBI for tentative phylogenetic identification. Pictured left are several isolates that were sequenced and classified.

Once each isolate received a tentative classification, they were struck together on petri dishes to attempt to force a interaction. When *B. pumilus* and *B. marisflavi* were crossed an inhibitory behavior was observed.

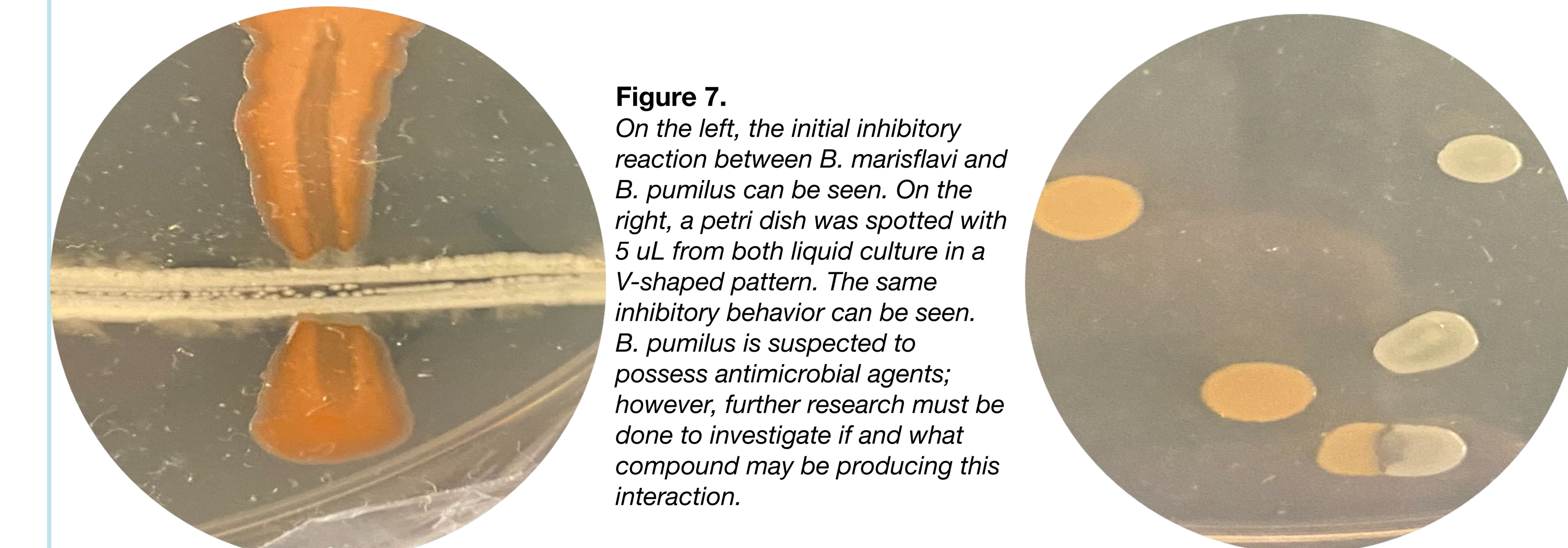
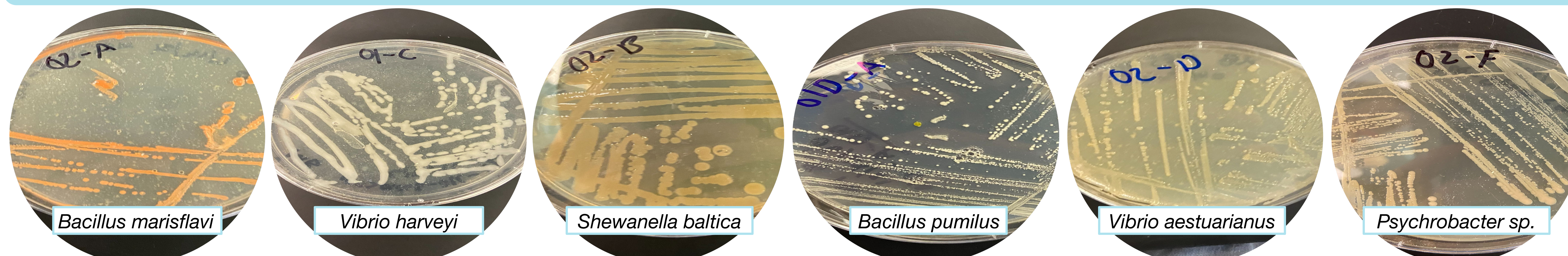


Figure 7.
On the left, the initial inhibitory reaction between B. marisflavi and B. pumilus can be seen. On the right, a petri dish was spotted with 5 uL from both liquid culture in a V-shaped pattern. The same inhibitory behavior can be seen. B. pumilus is suspected to possess antimicrobial agents; however, further research must be done to investigate if and what compound may be producing this interaction.

Microbial Ecology



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