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Kirby-Bauer Disc Diffusion Method Indicates Absence of Antimicrobial Properties in Ariolimax columbianus Mucus

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ABSTRACT

Antibiotic resistance is a rapidly accelerating epidemic demanding novel approaches. Gastropod mucus has been shown to possess antimicrobial properties and could potentially be used as an ingredient in antibiotic development. However, whether the mucus of *Ariolimax columbianus*, the banana slug, also displays antimicrobial properties is unknown. In this study, we investigated whether the mucus of *A. columbianus* is resistant to *Escherichia coli (E.coli)*, *Streptococcus aureus (S.aureus)*, and *Klebsiella pneumoniae (K.pneumoniae)*, three medically relevant strains of bacteria. Specimens were collected from a coniferous forest and isolated for downstream mucus extraction. We spread uniform concentrations of our bacteria on Mueller-Hinton agar plates and subjected them to a Kirby-Bauer disc diffusion test by treating them with either discs dipped in mucus or discs dipped in mucus and HBSS. Zones of inhibition did not form on the plates after subjecting the bacteria to either treatment. While this study was limited to a few taxa and one experimental approach, our study suggests that gastropod mucus may not have a generalized scope of antimicrobial activity. Rather, antimicrobial activity of mucus may be more specific to taxa encountered by the slugs in their redwood forest habitat. Our results can be used to refine mucus extraction methods for *A. columbianus* in future studies that seek to investigate the potential of mucus for biotechnological applications.

INTRODUCTION

The World Health Organization has identified antibiotic resistance as the greatest threat to technological development, international health, and food security. The ubiquitous use of antibacterial agents in clinical and agricultural settings to treat or prevent infections remains a driver for bacterial resistance (1). At the forefront of antibiotic consumption are livestock raised for food, with 73% of all antibiotics produced globally serving this purpose (2). Severe consequences result from a deluge of antibiotic administration, such as the pollution of aquatic areas, wastewater, and soil. The term "environmental resistome" has been widely used to refer to how human activities are increasing the probability of spreading pathogenic, antibiotic-resistant genes into the environment (3).

Ariolimax columbianus, the banana slug, is a beloved California native, boasting one of the largest body sizes of any terrestrial mollusc. German immigrants to California would gut *A. columbianus* like fish and fry them in batter, and they have been past staples of the Yurok tribe's diet. Throughout the 1980's, an annual festival at the Russian River celebrated banana slugs with cooking contests and slug races (4).

Pathogenic bacteria are becoming increasingly resistant to antibiotics. Many bacteria have evolved a selective stress response mechanism whereby a stress-inducing stimulus can trigger changes in gene expression patterns, favoring the production of enzymes and other proteins that increase survivorship. Bacteria that develop antibiotic resistance may increase their own fitness and others' by exchanging genetic information via mechanisms such as conjugation, transduction, and transformation. These processes allow such novel mutations to spread throughout a population (6).

There is a high demand for low-cost strategies and novel science-based ideas to combat pathogenic and antibiotic-resistant bacterial strains. Research and development of new antibiotics has waned over the years because more emphasis is placed on currently approved and commercialized antibiotics (7). Yet the prominence of multidrug-resistant bacteria remains an enormous public health risk that threatens the security of our nation's health care and economic system. As of 2020, the U.S. government has stepped in with a comprehensive antibiotic resistance plan that serves to promote active research into antimicrobial agents and preventative measures. This endeavor has led researchers to

investigate phyla such as Mollusca in their search for the answer to the antibiotic resistance epidemic.

Gastropods, members of the phylum Mollusca, produce mucus to aid in locomotion, reproduction, prevention of desiccation, predator repellence, and prevention of physical or chemical injuries. While their mucus is most commonly associated with epithelial regeneration and wound healing (8), it has many other biotechnological applications. The developments from implementing mucus treatments in medicine include surgical-grade glue (9), pain relievers such as Ziconotide (10), and drug delivery techniques (11). Untapped medical applications include topical cancer treatments in conjunction with nanoparticles of "oligodynamic noble metals" (12), chemotherapeutic and antidiabetic drugs (13), and antibiotics (14). Research into gastropod mucus for biotechnology has revealed key chemical constituents (8) that contribute to the inhibition of bacterial growth. Of interest, research with the mucus from the African giant snail was found to display antimicrobial peptides resistant to both gram-positive and gram-negative bacteria at room temperatures (8). Thus, mucus may provide a physical barrier to the microbial environment.

Despite the plethora of information available that suggests gastropod mucus is microbe-resistant, it remains unknown whether *A. columbianus* mucus exhibits the same bacterial resistance. It is possible that the mucus possesses antibacterial properties given the slugs' detritivorous diet and abundance in microbially diverse coniferous redwood forests. Thus, it might serve as a protective barrier against fungi, viruses, and bacteria. In this study, we ask whether *A. columbianus* mucus displayed antimicrobial properties when exposed to sustained, concentrated growth of three clinically relevant bacteria often targeted by conventional antibiotics: *E.coli, S.aureus, and K.pneumoniae*. We hypothesize that a noticeable marker of resistance, called the zone of inhibition, would develop on mucus-treated plates exposed to all three taxa. To observe this, we extract mucus from six *A. columbianus* specimens either directly or indirectly with immersion in HBSS (Hank's Balanced Salt Solution). HBSS is a biologically stable solution of sodium bicarbonate that will encourage mucus production without causing dehydration. To further stimulate mucus production with HBSS, slugs were gently rocked back and forth for a few minutes with HBSS solution. Next, we drench paper discs with the mucus and inoculate them on Mueller-Hinton agar plates alongside strains of E. coli, S. aureus, and K. pneumoniae. This microbiological assay, called the Kirby-Bauer disc diffusion test, is based on the idea that if bacteria do not grow within a certain distance of a treatment, then the treatment has antibacterial properties. The Kirby-Bauer method coupled with the protein-rich Mueller-Hinton agar medium is an efficacious way to quantitatively identify susceptibility of microorganisms to antibiotic treatments.

MATERIALS AND METHODS

Slug collection and maintenance

We collected six *A. columbianus* specimens from several patches of skunk cabbage in the Arcata Community Forest. They were kept together in an aerated lidded catering tray lined with wood, detritus and various foliage for two weeks. They were also given celery to supplement their diet and spritzed regularly with water to promote mucus secretion. After two weeks of experimentation, they were released back into the forest.

Spread plating

Broth cultures of *S. aureus, E. coli*, and *K. pneumoniae* were grown in nutrient-rich media overnight. We then used the spread plate technique to apply 250µL of

E. coli and *K. pneumoniae*, we then applied 500µL of *S. aureus,* to Mueller-Hinton agar plates while keeping our materials by a flame to ensure a sterile environment along with aseptic techniques between inoculations.

Mucus extraction & Kirby-Bauer disc diffusion test

We employed two different methods of mucus extraction: direct and indirect. In the direct extraction method, *A. columbianus* specimens were first isolated and rinsed with deionized water. We transferred the rinsed specimens into a sterilized glass container. Then, we used forceps to blot either the specimens themselves or their slime trails with sterile filter paper discs. Two replicate plates with four discs per plate were produced for each taxon. We sectioned our negative control plate in thirds and spread 100µL of a different bacterium on each section, applying a single untreated disc to each.

In the indirect extraction method, *A. columbianus* specimens were placed in 50mL of HBSS and gently rocked back and forth for 10 minutes. Then, discs were dipped in the mixture and applied to our plates. Altogether, we produced four replicates with three HBSS/mucus-dipped discs per plate. We also produced two negative control plates: one was segmented into thirds by bacterial taxon and treated with discs dipped in HBSS only, while the other was divided in half and subjected to our direct and indirect treatments.

We also experimented with two variations of the Kirby-Bauer procedure. For the first procedure, amounts of each strain specified above were transferred from concentrated broths and spread uniformly on our plates to promote the growth of

bacterial "lawns," or series of colonies that have merged to form mats of bacteria. Each set of plates was incubated at 37°C for 48 hours. Afterwards, we added our direct or indirect mucus treatments and incubated the plates again at 37°C for 24 hours. For the second procedure, we spread the same amount of each strain as above on our plates, but allowed the bacteria to grow alongside either one of the two treatments for 20 hours at 37°C. Photos were taken of all plates post-incubation.

RESULTS

In our first experimental attempt, we incubated our plates with each of the three taxa for 48 hours at 37°C, then plated our directly-treated discs and incubated at 37°C for another 24 hours. We noticed that all of the indirectly-treated discs were surrounded by a ring pattern **(Figures 2, 3, 4)**, while the directly-treated discs did not display this pattern **(Figures 5, 6, 7)**. We also noticed that a unique colonial morphotype developed on plates inoculated with *S. aureus*, but only around the indirectly-treated discs.

Our second experimental attempt consisted of concurrently incubating Muller-Hinton agar plates with *E. coli*, *S. aureus*, or *K. pneumoniae,* plus our two mucus extraction treatments. Bacterial rings formed around all indirectly-treated discs across taxa and their respective controls **(Figures 8, 9, 10)**. *E. coli* and *K. pneumoniae* appeared strikingly similar with respect to bacterial growth **(Figures 9 & 10)**. *S. aureus* was observed to have irregular and distinct bacterial colonies around the treated discs and throughout the plates **(Figure 8)**. Additionally, indirectly-treated discs plated on a bacteria-free control plate exhibited the same colonial morphologies **(Figure 15)**. Next, all taxa were grown alongside directly-treated discs. *E. coli* and *K. pneumoniae* again grew to similar densities **(Figure 13, 14)**. *S. aureus* grew minimally in comparison despite a greater amount of bacteria being inoculated for *S. aureus* plates **(Figure 12)**. Surprisingly, many small and distinct bacterial colonies had grown around our directly-treated discs alongside *S. aureus* **(Figure 12)**. Negative control discs grown alongside *S. aureus* did not have these bacterial colonies **(Figure 11)**. Additionally, these colonies were absent from our negative control M-H agar plate subjected to directly-treated discs **(Figure 15)**.

Towards the end of our experiment, we asked whether the HBSS may be playing a role in the colony morphologies observed for our indirectly-treated discs growing alongside *S. aureus*. We incubated HBSS discs without mucus on an M-H agar plate overnight. Unexpectedly, we observed colony morphologies around the HBSS discs (Figure 15). Table 1 displays a summarization of our results.

DISCUSSION

Our results suggest that growth of *S. aureus, K. pneumoniae, & E. coli* was not inhibited by the presence of *A. columbianus* mucus as expected. Rather, the mucus may actually promote

Figure 1.
Negative control plate for indirect treatments. Each bacterial taxon (E. coli, K. pneumoniae, and S. aureus) was inoculated and incubated at 37°C for 72 hours on our untreated negative Mueller-Hinton (M-H) agar control plate.

Figure 2.
Series of spread plates inoculated with S. aureus (A-C). Three replicates of S. aureus spread on M-H agar plates & treated with indirectly-extracted mucosal discs. Plates incubated for 70 hours at 37°C.

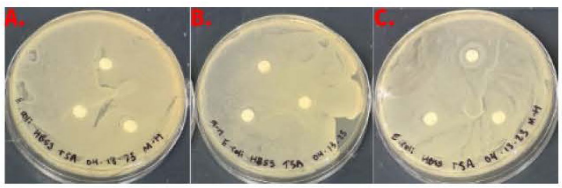


Figure 3.
Series of spread plates inoculated with K. pneumoniae (A-C). Three replicates of K. pneumoniae spread on M-H agar plates & treated with indirectly-extracted mucosal discs. Plates incubated for 70 hours at 37°C.

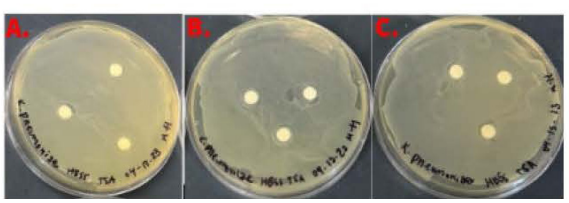


Figure 4.
Series of spread plates inoculated with S. aureus (A-C). Three replicates of S. aureus spread on M-H agar plates & treated with indirectly-extracted mucosal discs. Plates incubated for 70 hours at 37 °C.

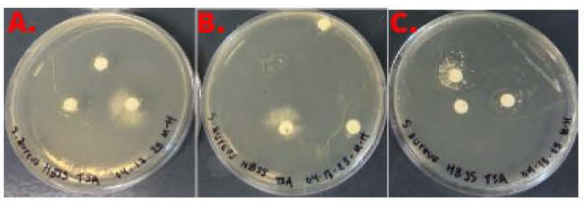


Figure 5.
Series of spread plates inoculated with S. aureus. S. aureus negative control plate not subjected to mucosal treatment (A). Four replicate spread plates of S. aureus treated with directly-extracted mucus discs (B-E). Plates incubated for 70 hours at 30°C

Figure 6.
Series of spread plates inoculated with K. pneumoniae. K. pneumoniae negative control plate not subjected to mucosal treatment (A). Four replicate spread plates of K. pneumoniae treated with directly-extracted mucus discs (B-E). Plates incubated for 70 hours at 37°C.

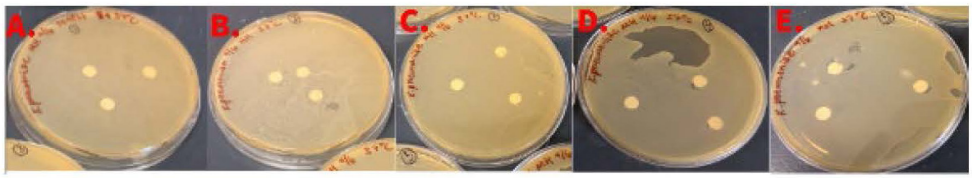


Figure 7.
Series of spread plates inoculated with E. coli. E. coli negative control plate not subjected to mucosal treatment (A). Four replicate spread plates of E. coli treated with directly-extracted mucus discs (B-E). Plates incubated for 70 hours at 37°C.

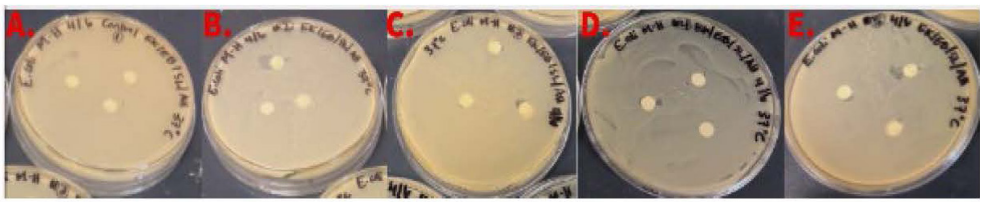


Figure 8.
Series of spread plates inoculated with S. aureus. Negative control of S.aureus spread on M-H agar with indirect-extracted mucus discs (A). Four spread plate replicates of S. aureus treated with indirectly-extracted mucus discs (B-E). Plates incubated for 20 hours at 37°C

Figure 9.
Series of spread plates inoculated with K. pneumoniae. Negative control of K. pneumoniae spread on M-H agar with indirect-extracted mucus discs (A). Four replicates of K. pneumoniae spread on M-H plates and subjected to indirectly extracted mucus-treated discs (B-E). Plates incubated for 20 hours at 37 °C

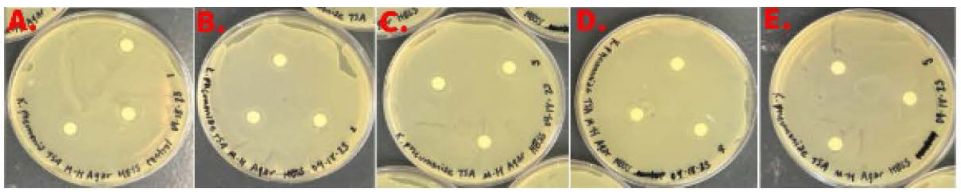


Figure 10.
Series of spread plates inoculated with E. coli. Negative control of E. coli spread on M-H agar with indirect-extracted mucus discs (A). Four replicates of E. coli spread on M-H plates and subjected to indirect mucus-treated discs (B-E). Plates incubated for 20 hours at 37 °C.

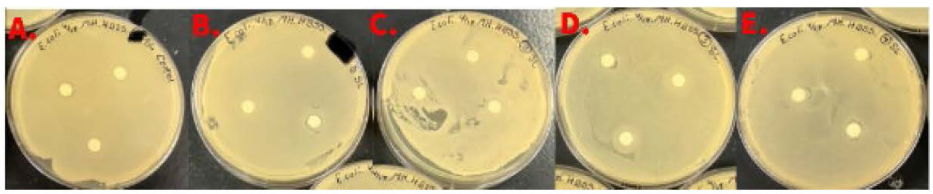


Figure 11.
Negative control M-H agar plate with inoculation of E. coli, K. pneumoniae, & S. aureus with untreated discs. Incubated at 37°C for 20 hours.

Figure 13.
K. pneumoniae inoculated on M-H agar plates and treated with directly- extracted discs. Incubated at 37°C for 20 hours

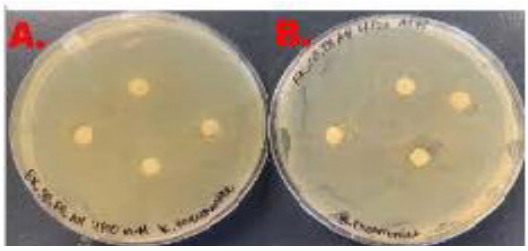


Figure 14.
E. coli inoculated on M-H agar plates and treated with directly extracted discs. Incubated at 37°C for 20 hours.

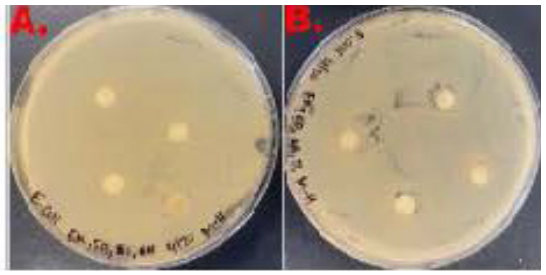
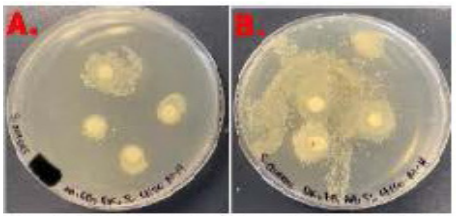


Figure 15.
Negative control M-H agar plate. Indirectly extracted treatment (left) and directly extracted treatment (right).

Figure 16.
Indirect-treated discs on M-H agar plate incubated at 37°C for 20 hours. Clear indication of contamination, as growth around the discs was not expected.

Figure 12.
S. aureus inoculated on M-H agar plates and treated with directly- extracted discs. Incubated at 37°C for 20 hours.



growth of these strains of bacteria, or not interact with them at all. While mucus may serve as a physical protective barrier for *A. columbianus* in the wild, we can neither conclude that it protects the organism from environmental pathogens, nor that it has any downstream medical applications for the taxa tested.

Throughout this study, we observed intriguing colonial growth on plates inoculated with *S. aureus* when treated with both directly- and indirectly-extracted mucosal discs. We can attribute this to contaminated HBSS mixing with our indirectly-extracted mucus for some plates; however, unexpected colonial abundance on *S. aureus* plates treated with directly-extracted mucosal discs can be interpreted in two ways. One, properties within *A. columbianus* mucus may encourage microbial growth factors required for *S. aureus* proliferation; or two, it is possible that bacteria within the mucosal microbiome may be growing in response to associations with *S. aureus*. It is likely that these bacterial colonies would have been observed in *E. coli* and *K. pneumoniae* if it were not for the excessive growth of these taxa. Additionally, it is not known whether endogenously-produced bacteria are secreted in *A. columbianus* mucus. It is possible that these endogenous bacteria may have been represented on our plates without our knowledge.

In the future, we suggest conducting a similar experiment but with bacterial taxa that *A. columbianus* regularly come into contact with within their environment. Of considerable importance, antimicrobial peptides present within the mucus may have been rendered inactive or destroyed at our incubation temperature of 37°C. Therefore we advise careful consideration of incubation temperatures to ensure mucosal properties are not being compromised for bacterial growth. All in all, this study paves the way towards understanding the role of *A. columbianus* mucus as it pertains to its environment.

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Table 1.
Summarization of results.

Figure #	Treatment	Taxa/Taxon	Replications	Inhibition
Figure #1	Control, no treatment	*S. aureus, K. pneumoniae, E. coli*	N/A	No
Figure #2	Indirect-Extraction	*S. aureus*	3 replicates	No
Figure #3	Indirect-Extraction	*K. pneumoniae*	3 replicates	No
Figure #4	Indirect-Extraction	*E. coli*	3 replicates	No
Figure #5	Direct-Extraction	*S. aureus*	Control + 4 replicates	No
Figure #6	Direct-Extraction	*K. pneumoniae*	Control + 4 replicates	No
Figure #7	Direct-Extraction	*E. coli*	Control + 4 replicates	No
Figure #8	Indirect-Extraction	*S. aureus*	Control + 4 replicates	No
Figure #9	Indirect-Extraction	*K. pneumoniae*	Control + 4 replicates	No
Figure #10	Indirect-Extraction	*E. coli*	Control + 4 replicates	No
Figure #11	Control, no treatment	*S. aureus, K. pneumoniae, E. coli*	N/A	No
Figure #12	Direct-Extraction	*S. aureus*	2 replicates	No
Figure #13	Direct-Extraction	*K. pneumoniae*	2 replicates	No
Figure #14	Direct-Extraction	*E. coli*	2 replicates	No
Figure #15	Indirect-Extraction	N/A	N/A	No
Figure #16	HBSS-treated	N/A	N/A	No

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