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Article

Effect of Gram-positive bacteria on antibiotic resistance in Gram-negative bacteria

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SUMMARY

Antibiotics are one of the most common treatments for bacterial infections, but the emergence of antibiotic resistance is a major threat to the control of infectious diseases. Many factors contribute to the development of antibiotic resistance. One is bacterial conjugation from Gram-positive to Gram-negative bacteria where there is a transfer of resistance genes from Gram-positive to Gram-negative bacteria that could increase antibiotic resistance in the latter. In light of these observations, we decided to test whether Gram-negative bacteria that came into contact with Gram-positive bacteria had a higher resistance to the antimicrobial properties of spices than Gram-negative bacteria that did not come into contact with Gram-positive bacteria. We hypothesized that Gram-negative bacteria that had been exposed to Gram-positive bacteria would be more resistant to antibiotics than unexposed Gram-negative bacteria. To test our hypothesis, we cultured and subcultured lettuce (Gram-negative) bacteria and forearm (Gram-positive) bacteria, as well as lettuce bacteria that encountered forearm bacteria, on agar plates using powdered ginger, which has previously been shown to have antimicrobial properties to assess antibiotic resistance. We found that lettuce bacteria that encountered forearm bacteria were indeed more resistant to ginger since they showed more growth than lettuce bacteria. These results indicate that Gram-positive bacteria may transfer their resistance genes to Gram-negative bacteria, resulting in increased antibiotic resistance. If thousands of Gram-positive bacteria become antibiotic-resistant and pass those genes to Gram-negative bacteria found in the human body, it can make bacterial infections more difficult to treat.

INTRODUCTION

Bacterial infections are the cause of many infectious diseases and are one of the greatest contributors to death and illness (1). Antibiotics are a common treatment for bacterial infections; however, the issue of antibiotic resistance continues to threaten the control of infectious diseases (2). For instance, in the United States alone, approximately 2.8 million antibiotic-resistant infections occur annually, leading to an estimated 35,000 deaths every year (3). Furthermore, antibiotic resistance in bacteria varies depending on whether the bacteria is Gram-positive, which is characterized by a thick peptidoglycan cell wall, or Gram-negative, which has a cell wall consisting of a thin layer of peptidoglycan and an outer membrane of lipopolysaccharide (1). The different membrane compositions of the two Gram types can impart unique antibiotic resistance mechanisms to each (1). For instance, resistance to β-lactams in Gram-negative bacteria is mainly accomplished by the production of β-lactamases; however, Gram-positive bacteria tend to modify the β-lactam target site, penicillin-binding proteins located in the bacterium's plasma membrane, instead. The different mechanisms are attributed to differences in cell wall structure: Gram-negative bacteria are able to control entry of molecules like β-lactams into the cell because there is an outer membrane protecting the inner plasma membrane. β-lactamases present in the periplasmic space between the outer and inner membrane can help destroy the β-lactams before they reach their target site. However, the absence of this protection in Gram-positive bacteria has likely caused bacteria of this Gram type to develop a different mechanism to combat β-lactams, demonstrating how membrane composition can be a factor in explaining differences in antibiotic resistance mechanisms for different bacteria (4). Antibiotic resistance in either type of bacteria is dangerous because of its direct impact on the lethality of infections, as well as the cost of healthcare (5, 6). A lack of effective treatments in treating antibiotic-resistant bacteria can lead to longer hospital stays, increased use of hospital resources, and excess surgery, which increase the costs of treating bacterial infections (7).

Many factors contribute to the development of antibiotic resistance. One prominent contributor is bacterial conjugation, which is the process by which DNA is transferred from one bacterial cell (the donor) to another bacterial cell (the recipient) through direct cell-to-cell contact (8). Bacterial conjugation can often lead to a transfer of antibiotic-resistance genes and can occur in both Gram-negative and -positive bacteria, even those of different Gram types. Gram-negative bacteria often acquire antibiotic resistance determinants via conjugation with Gram-positive donors (9). Although trans-Gram bacterial conjugation can be potentially hazardous, few studies have focused on this issue (10). Further, while numerous studies have focused on conjugation in Gram-negative bacteria, these mechanisms are less studied in Gram-positive bacteria (10).

In light of these observations, we decided to conduct a

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study testing whether Gram-negative bacteria that came into contact with Gram-positive bacteria had higher antibiotic resistance than Gram-negative bacteria that did not come into contact with Gram-positive bacteria. We hypothesized that Gram-negative bacteria exposed to Gram-positive bacteria would be more resistant to antibiotics than unexposed Gram-negative bacteria as a result of potential conjugative transfer of antibiotic resistance genes from Gram-positive to Gram-negative bacteria. To test this, we used lettuce and forearm skin bacteria, known sources of Gram-negative bacteria (specifically, *E. coli*) and Gram-positive bacteria (specifically, *S. aureus*), respectively (11, 12). We also used powdered ginger as our antibiotic. Spices have previously been shown to demonstrate antibacterial properties. Ginger in particular has exhibited anti-microbial activity before, including against *E. coli* and *S. aureus*, as it contains various biologically active constituents that can have antimicrobial properties (13-16). The effectiveness of ginger's antimicrobial properties varies between bacterial species, with Gram-negative bacteria being more resistant to ginger extract than Gram-positive (16, 17). Ginger extract can destroy the cell membrane of pathogens, resulting in cell content leakage and inhibition of important endoenzymes' activities, such as those of succinate dehydrogenase and alkaline phosphatase, that are key for the growth of bacteria (18). We assessed whether lettuce bacteria that encountered forearm skin bacteria had more bacterial growth compared to unexposed lettuce bacteria, which is indicative of increased antibiotic resistance. We found that the exposed Gram-negative bacteria were indeed more resistant to ginger than the unexposed one. These results indicate that Gram-positive bacteria were possibly able to transfer its resistance genes to Gram-negative bacteria as hypothesized. The potential ease with which inter-Gram conjugation can occur poses a threat to the control of antibiotic resistance.

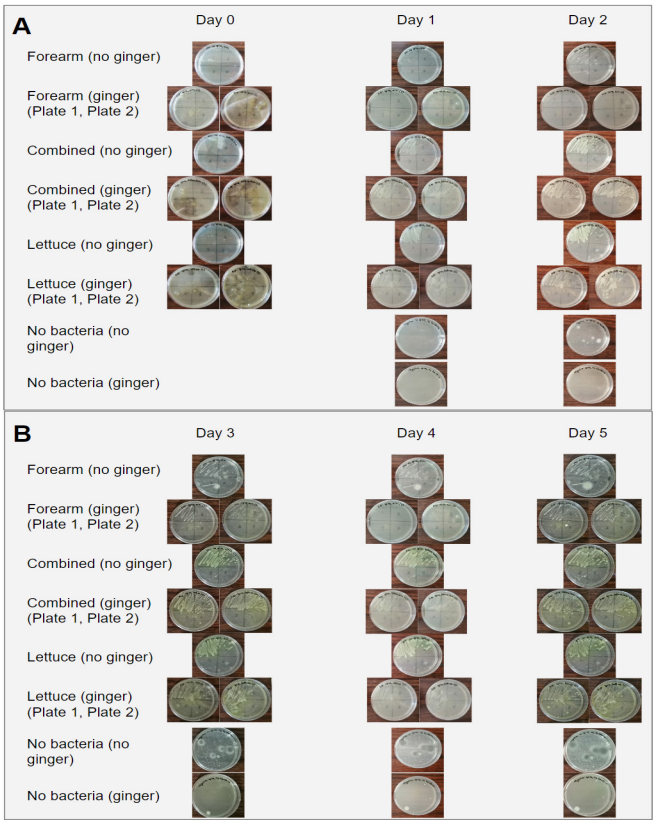

Figure 1: Antibiotic resistance in bacterial plates. Images of forearm, lettuce, and combined bacteria plates. Panel A shows the images from Day 0 to Day 2, and Panel B shows the images from Day 3 to Day 5. Forearm, lettuce, and combined bacteria were grown on LB agar plates and their growth was measured over five days. The positive controls are the "Lettuce (ginger)" and "Forearm (ginger)" plates. The negative controls are the "No bacteria (no ginger)", "No bacteria (ginger)", "Lettuce (no ginger)", and "Forearm (no ginger)" plates. The experimental plates are the "Combined (no ginger)" and "Combined (ginger)" plates. Negative controls are shown for Day 1, 2, 3, 4 and 5; no images for the "No bacteria (no ginger)" and "No bacteria (ginger)" plates were recorded for Day 0.

RESULTS

To test our hypothesis that Gram-negative bacteria exposed to Gram-positive bacteria would have greater antibiotic resistance than unexposed Gram-negative bacteria, we cultured lettuce (hypothetically Gram-negative) bacteria, forearm (hypothetically Gram-positive) bacteria, and lettuce bacteria that had encountered forearm bacteria ("combined" bacteria) on agar plates (**Figure 1**).

Forearm Bacteria

To verify that the forearm bacteria were Gram-positive, we analyzed colony morphology. The forearm bacteria were white, with small round colonies and a distinct border on each. The shape was convex (raised). Although this was not in line with the characteristics of *S. aureus* on nutrient agar cultures, this matched descriptions of *L. plantarum*, another species of Gram-positive bacteria that can be found in food and the human body (19).

The forearm bacteria unexposed to ginger grew on average more than the exposed bacteria. On Day 5, the area percentage of forearm bacteria unexposed to ginger was 4.27% and the area percentage of forearm bacteria exposed to ginger was 3.12%. The exposure to ginger resulted in a 26.9% decrease in area percentage of forearm bacteria (**Figure 2**). The average ratio of bacteria with ginger to bacteria without ginger increased continuously from Day 1 to Day 5 (0.44 to 0.73). This means that on Day 1, the number of bacteria exposed to ginger was about 44% to the number of bacteria unexposed to ginger, but on Day 5, the number of bacteria exposed to ginger was 73% to the number of the bacteria unexposed to ginger.

The rate of growth of the forearm bacteria with ginger

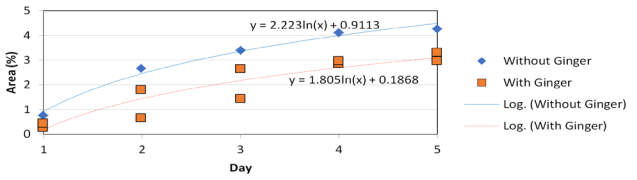

Figure 2: Bacterial growth in ginger-treated and non-treated forearm bacteria. Scatter plot showing percent area of plate covered by forearm bacterial growth each day from Day 1 to Day 5. Although individual data points were shown for each replicate (one for non-treated bacteria and two for ginger-treated bacteria), the fitted curves correspond to the average percent area for each day. Forearm bacteria was grown in agar plates, some with ginger (positive control) and one without ginger (negative control).

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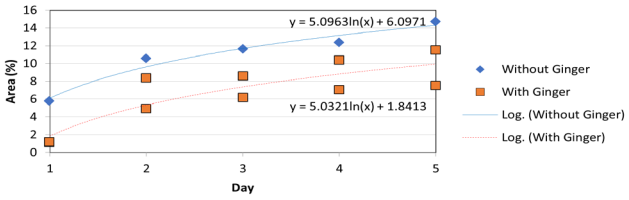

Figure 3: Bacterial growth in ginger-treated and non-treated lettuce bacteria. Scatter plot showing percent area of plate covered by lettuce bacterial growth each day from Day 1 to Day 5. Although individual data points were shown for each replicate (one for non-treated bacteria and two for ginger-treated bacteria), the fitted curves correspond to the average percent area for each day. Lettuce bacteria were plated on agar plates both with and without ginger.

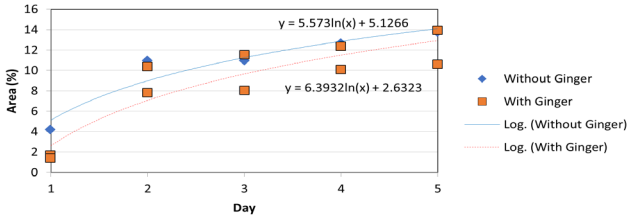

Figure 4: Bacterial growth in ginger-treated and non-treated combined bacteria. Scatter plot showing percent area of plate covered by combined bacteria growth each day from Day 1 to Day 5. Although individual data points were shown for each replicate (one for non-treated bacteria and two for ginger-treated bacteria), the fitted curves correspond to the average percent area for each day. Lettuce bacteria that had come into contact with forearm bacteria during initial culturing (combined bacteria) were plated on agar plates both with and without ginger.

(1.81%/day) appeared to be slower than without ginger (2.22%/day) (**Figure 2**); however, we were not able to conduct statistical tests to confirm this due to a lack of replicates.

Lettuce Bacteria

To verify that the lettuce bacteria were Gram-negative, we analyzed colony morphology. The lettuce bacteria were opaque yet slightly translucent with a whitish, yellow-greenish tint. Compared to the forearm bacteria colonies, the lettuce bacterial colonies were much larger and appeared moist and smooth. These are all characteristics of Gram-negative *E. coli* grown on nutrient agar cultures (20).

The lettuce bacteria unexposed to ginger grew more than the one exposed to ginger, when comparing over any day from Day 1 through Day 5 (**Figure 3**). For example, on Day 5, the area percentage of lettuce bacteria unexposed to ginger was 14.72% and the area percentage of lettuce bacteria exposed to bacteria was 9.50%. The exposure to ginger resulted in a 35.5% decrease in area percentage of lettuce bacteria (**Figure 3**). The average ratio of bacteria exposed to ginger to unexposed to ginger was 0.65.

Interestingly, the lettuce bacteria exposed to ginger and unexposed to ginger showed similar growth rates. Lettuce bacteria with ginger showed a growth of 5.03%/day, and without ginger showed a growth of 5.10%/day (**Figure 3**). However, the Day 1 plate areas had a 69.8% difference. On Day 1, Lettuce bacteria exposed to ginger had a plate area of 1.84%, whereas lettuce bacteria unexposed to ginger had a plate area of 6.10%.

Combined Bacteria

The combined bacteria (Gram-negative bacteria exposed to Gram-positive bacteria) cultures looked visually quite similar to the lettuce bacteria, indicating that it is also E.coli (Gram-negative) (**Figure 1**).

As observed in forearm bacteria, the combined bacteria unexposed to ginger also had a larger area percentage than combined bacteria exposed to ginger. On Day 5, the area percentage of lettuce bacteria unexposed to ginger was 13.68% and the area percentage of lettuce bacteria exposed to ginger was 12.24%. The exposure to ginger resulted in a 10.5% decrease in area percentage of lettuce bacteria (**Figure 4**). The average ratio of bacteria unexposed to ginger to exposed to ginger was 0.77.

Unlike forearm bacteria, the combined bacteria saw a higher growth rate in the bacteria exposed to ginger than unexposed to ginger. The rate of growth exposed to ginger was 6.39%/day, whereas the rate of growth unexposed to ginger was 5.57%/day (**Figure 4**). The rate of growth for combined bacteria with ginger is approximately equal to the rate of growth for forearm bacteria with ginger (1.81%/day) plus the rate of growth for lettuce bacteria with ginger (5.03%/day).

DISCUSSION

Our experiment attempted to further investigate the processes of inter-Gram gene transfer, and particularly the effectiveness of inter-Gram transfer in increasing antibiotic resistance in Gram-negative bacteria. All bacteria have a property called Gram type that refers to the composition of the cell wall and cell membrane of the bacteria, which imparts unique mechanisms of antibiotic resistance. However, the important phenomenon of inter-Gram transfer is relatively more novel and less studied – an issue we attempted to address in this research (21). Our findings support the hypothesis that combined (Gram-negative lettuce bacteria that had been exposed to Gram-positive forearm bacteria) bacteria are more resistant to the antibacterial properties of ginger than Gram-negative lettuce bacteria alone. The average ratio of bacterial growth exposed to ginger to unexposed to ginger was higher in the combined bacteria than it is in the forearm bacteria. The rate of growth for the combined bacteria exposed to ginger was also greater than those of either the forearm or lettuce bacteria. Therefore, the combined bacteria demonstrated potentially greater resistance to the antibacterial properties of ginger than lettuce or forearm bacteria. These findings potentially indicate that the Gram-negative bacteria underwent gene transfer (possibly by bacterial conjugation) of antibiotic resistance genes from the Gram-positive bacteria. This would also be corroborated by some of the visual differences between the combined bacteria and lettuce bacteria, which might be attributed to its encounter with the forearm (Gram-positive) bacteria. However, future research is needed to verify these findings.

The change in percentage area each day decreased across all bacterium types, indicating that growth slowed as time progressed (**Figure 5**). However, all bacterium types also showed an increase in the ratio of ginger to no ginger as time progressed (**Figure 6**). A higher ratio signifies that the amount

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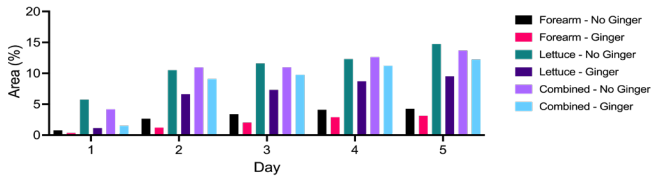

Figure 5: Plate area of all bacterium types and spice conditions. Bar graphs showing percent area of plate covered by lettuce, forearm, and combined bacteria with and without spice over all five days.

of bacteria exposed to ginger was closer to the amount of bacteria unexposed to ginger (normal growth), illustrating that the bacteria was not as affected by the presence of ginger as expected. Our data indicated that as time progressed, all bacteria exposed to ginger approached their normal growth rates.

Interestingly, the lettuce bacteria had similar growth rates both with and without ginger. However, the Day 1 plate areas were quite different; the Day 1 plate area for lettuce bacteria with ginger was much lower than the one without ginger. This indicates that the spice did not affect the rate of growth so much as it caused the lettuce bacteria to grow on a smaller scale. In other words, the ginger reduced the starting (Day 1) concentration, likely by killing some of the viable cells before they could grow. While it is possible that there were different starting amounts of bacteria, the concentrations of the subcultures for each bacteria type did not seem particularly different from each other visually, and therefore any differences in starting amount are unlikely to account for the significant difference between the growth of the lettuce bacteria unexposed to ginger versus exposed to ginger.

Comparing lettuce and forearm bacteria, we see that the lettuce bacteria had a larger average ratio of ginger to no ginger over the span of five days, illustrating that the lettuce bacteria had greater resistance to ginger than forearm bacteria. Lettuce bacteria also had a greater rate of growth. Further, while the rate of growth for the forearm bacteria exposed to ginger was less than its rate of growth unexposed to ginger, the rate of growth for the lettuce bacteria was unaffected. These observations indicated a potentially greater resistance towards ginger in the lettuce bacteria compared to forearm bacteria, corroborating previous research that found that Gram-negative bacteria are more resistant to ginger extracts than Gram-positive bacteria.

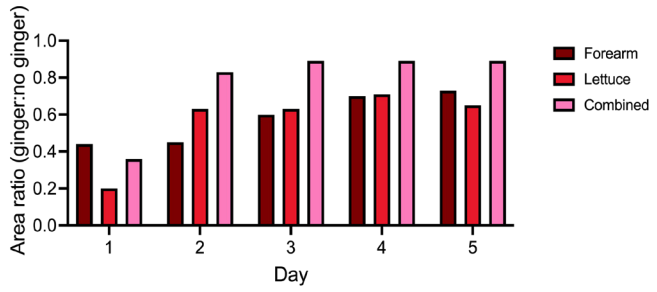

Figure 6: Plate area ratio of ginger to no ginger. Bar graph comparing the ratios of spice to no spice for each bacterium type (forearm, lettuce, and combined). Lettuce, forearm, and combined bacteria were plated on agar plates and the area ratios between plates with ginger and plates without ginger were recorded.

A possible explanation for these results is that there may be an overlap in the antibiotic resistance genes present in both Gram-positive and Gram-negative bacteria. While the forearm bacteria might be less resistant to antibiotics than lettuce bacteria, the combined bacteria still benefits from the genes transferred by the forearm bacteria, resulting in increased antibiotic resistance. This would also explain our observation that the rate of growth for combined bacteria is approximately equal to, but slightly less than, the rate of growth for forearm bacteria plus the rate of growth for lettuce bacteria. The resistance of the combined bacteria is equal to the lettuce bacteria plus what was transferred from the forearm bacteria, minus the overlap in their antibiotic resistance genes. So, while Gram-negative bacteria may have had more resistance than the Gram-positive bacteria, the combined bacteria still benefited from the transferred Gram-positive resistance to become more resistant. If our results are more definitively proven in the future, this would be an additional concern for mitigating the current increase in antibiotic resistance.

These results, however, may have been affected by potential errors while conducting the experiment. Bacteria were not sampled and cultured at the same time, which means differences in temperature and weather may have influenced the bacterial growth. The experiment was also not conducted in a lab, but rather in the home setting, which made it difficult to standardize the starting concentrations of the bacteria during subculturing as well as conduct Gram stains to determine the Gram-type of the plated cultures. As a result, comparisons between the different bacterium types might have been skewed by the different starting concentrations. Nonetheless, the subculture concentrations were not significantly different visually, as mentioned previously. With the home setting, we also were not able to standardize the temperature at which the bacteria grew.

Because of a lack of bacterial growth in some of the agar plates, the experiment had to be repeated. The lack of growth may have been due to a timing issue: when preparing the bacteria for culturing, the centrifuge tubes were left for approximately 86 hours instead of 48-72 hours. This may have caused the bacteria to die from starvation because there were not enough nutrients in the tryptic soy broth in the tube to sustain life.

There were also a few limitations regarding the agar plates. Agar was microwaved in an uncapped bottle, exposing it to potential contaminants in the air. This may explain the growth on the negative controls in the subculturing process (**Figure 1**). The contaminants that grew on the negative controls were not visible on the experimental plates; however, it is possible that there were indeed contaminants trapped in the agar, but they simply did not grow colonies because only a small fraction of cells is culturable on plates (22, 23). Despite not being visible, the contaminants could still interact with the bacteria samples and affect bacterial growth. Also, the amount of agar in each plate varied. Some agar plates also developed bubbles in the agar and uneven or unsmooth surfaces. The presumptions that Gram-negative (*E. coli*) bacteria could be found in lettuce and Gram-positive (*S. aureus* that turned out to potentially be *L. plantarum*) bacteria could be found on the skin formed the basis of the experiment methods (11, 12). However, no Gram stain was performed, and therefore the Gram type of the sampled bacteria could

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not be confirmed. If this experiment were to be conducted again in a lab setting, we would use a Gram stain to ensure we are using Gram-negative and Gram-positive bacteria.

Other limitations include the choice of measure for antibiotic resistance (i.e. ginger). This experiment also only used powdered ginger, which had been previously proven to have various antibacterial properties (13). Future experiments might compare the effects of powdered versus fresh or dried spices on bacterial growth for Gram-positive and Gram-negative bacteria, or test the effects of using different spices and other antibiotics. Another limitation was our inability to confirm that bacterial conjugation occurred, so some of our combined bacteria may not have received any genes from the Gram-positive bacteria.

In this experiment, bacterial colonies were counted using ImageJ, a Java-based software developed by the National Institutes of Health (NIH). The software requires settings to be manually adjusted in order to detect bacterial growth on the plates. Therefore, the measured plate areas would vary depending on the settings, decreasing the accuracy of measurements. For example, it was found that combined bacteria had a higher growth rate exposed to ginger versus unexposed to ginger. This is unlikely to have actually happened considering previous research on the antibacterial effects of ginger, and is more likely an indicator of measurement imprecision. A more definitive method of measuring plate areas or counting bacterial colonies would be preferred.

We also were not able to perform any statistical tests since we did not have a sufficient quantity of data. This also highlights the uncertainty of our conclusions. Future research should be conducted replicating this experiment to confirm our findings.

In the past, bacterial conjugation was thought to occur only between closely-related bacteria (9). However, Courvalin's study and many following it have demonstrated that trans-Gram conjugation is indeed possible and reveals bacteria's exceedingly broad host range. Trans-Gram conjugation is potentially dangerous because of its contribution to increased antibiotic resistance in bacteria. For instance, animals have an abundance of Gram-positive bacteria; in pet animals, wildlife, and livestock, there are significantly more Gram-positive bacteria than Gram-negative bacteria (24, 25). Animals, especially farm animals, are frequently exposed to a number of antibiotics, which has contributed to antibiotic resistance in bacteria associated with animals (26). If Gram-positive bacteria in animals develop antibiotic resistance, it may be passed onto Gram-negative bacteria in humans, of which plenty can be found in the body and especially the gastrointestinal tract (27). This would make bacterial infections harder to treat in humans with currently available antibiotics. Because of this, concerns have been voiced regarding the use of antibiotics in animal feed (28).

Although this experiment was conducted in the home setting, which may have led to experimental errors, it also demonstrated that trans-Gram conjugation may be able to occur in a "natural" environment and not only in a lab setting. This makes trans-Gram conjugation even more unpredictable because of how easily it can occur in different environments.

Finally, this experiment studied the effects of conjugation between a Gram-positive donor and Gram-negative recipient. However, it is also possible for the opposite process (Gram-negative donor and a Gram-positive recipient) to occur (29). In fact, because Gram-negative bacteria had higher antibiotic resistance than Gram-positive bacteria, this type of trans-Gram conjugation might be even more harmful because more genes can be transferred (30).

MATERIALS AND METHODS

Preparing Bacteria for Culturing

Samples were taken from a leaf of store-bought lettuce for the Gram-negative bacteria sample and the skin of both forearms of one individual for the Gram-positive bacteria sample. We used tryptic soy broth (Beckton, Dickinson, Cat# 257107) to prepare our samples. Cotton swabs were dipped into Ultrapure water before swabbing the area of interest (lettuce or forearm) three to four times. For the negative control, nothing was swabbed. The swabs were dipped into centrifuge tubes filled with tryptic soy broth. The tubes were then capped and left to sit at room temperature for two to three days.

Initial Culturing

Initial culturing of the bacteria used three agar plates: one containing only lettuce bacteria, one containing only forearm bacteria, and a "combined plate" that had lettuce bacteria on the right side and forearm bacteria on the left.

Agar (Sigma-Aldrich, Cat# 05040) was melted by microwaving twice for 30-50 seconds before being poured into the petri dishes. The agar was poured just enough to fill the bottom of the plate. Any bubbles and uneven surfaces were left untreated because they were minor issues. The agar plates were covered and left to sit for at least two hours before use.

After cooling, the bacteria samples from the centrifuge tubes were transferred to the three agar plates for culturing. A Pasteur pipette was used to transfer a quarter-sized sample of the forearm bacteria to the forearm bacteria plate and a smaller amount to the left side of the combined plate. A new pipette was used to transfer similar amounts of lettuce bacteria to the lettuce bacteria plate and the right side of the combined plate. The bacteria were then spread on the plates using two L-spreaders: one for spreading forearm bacteria and one for spreading lettuce bacteria. The latter L-spreader was also used to mix the bacteria near the center of the combined plate where the two bacteria samples met to give a slight overlap. This was done so that the Gram-negative bacteria could come into contact with the Gram-positive bacteria; the L-spreader allowed for a more consistent overlap along the diameter of the plate, as opposed to a pipette, for example. Afterwards, the plates were covered, and the lids were taped to secure them in place. All three agar plates were then stored at room temperature for three to four days.

Preparing Bacteria for Subculturing

To prepare for subculturing, a single colony from each agar plate was transferred to centrifuge tubes. Tryptic soy broth (3 mL) was transferred to three new centrifuge tubes using a Pasteur pipette. Using a scoopula, one colony from each agar plate was transferred into the centrifuge tubes. For the lettuce bacteria and forearm bacteria plates, a random colony was taken. For the combined plate, a single colony was taken from the lettuce bacteria side near the border where it had come into contact with the forearm bacteria side (**Figure 7**). This was used as the sample of Gram-negative bacteria that had

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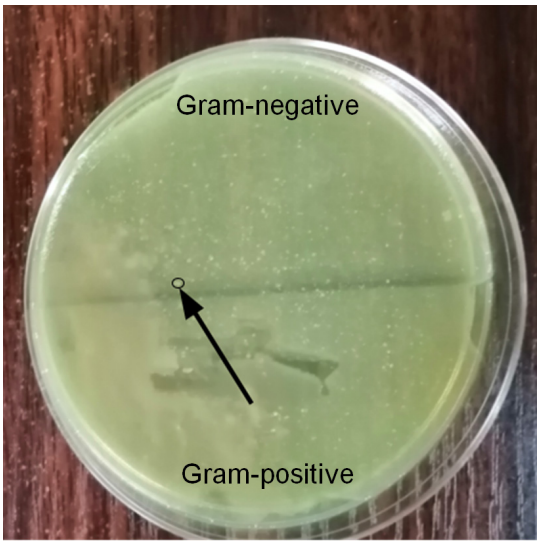

Figure 7: Selection of a Gram-negative bacteria colony that had come into contact with Gram-positive bacteria. Image of the combined plate during initial culture. The bottom half of the plate contains Gram-positive bacteria and the top half contains Gram-negative bacteria grown for 3-4 days. The chosen colony is indicated with an arrow.

been exposed to Gram-positive bacteria.

After the colonies were transferred to the centrifuge tubes and mixed with the soy broth inside, the tubes were capped and stored at room temperature for two to three days.

Subculturing

For subculturing, 11 agar plates were used. For the negative controls, one plate unexposed to bacteria and ginger and one plate with no bacteria but exposed to ginger were prepared, where ginger was used as our source of antibiotics. Additional negative controls were one plate with only lettuce bacteria and another plate with forearm bacteria. For positive controls, two plates with lettuce bacteria exposed to ginger and two plates with forearm bacteria exposed to ginger were prepared. For the experimental plates, one plate with combined bacteria and two plates with combined bacteria exposed to ginger were prepared.

To prepare the subculture agar plates, a new centrifuge tube was prepared first. A small amount of ginger was poured into the bottom (about 0.5-1g, following instructions from JEI's Mini-PhD Program bacteria protocol). Agar was then microwaved three times for 30 seconds each and poured into the centrifuge tube. The tube was shaken repeatedly until its contents were completely mixed. The ginger-infused agar in the tube was then poured into its designated plates. Regular, non-spice-infused agar was used for the other plates. The agar plates were left to sit for at least two hours.

After the agar plates had cooled, the three subculture bacteria samples were first diluted before being transferred to the plates. To dilute the samples, three new centrifuge tubes were prepared. Using a Pasteur pipette, three mL of tryptic soy broth were transferred to each tube. Three drops of each subculture sample were transferred to the new centrifuge tubes. The new tubes containing the diluted bacteria samples were shaken repeatedly to mix the bacteria.

The diluted subculture bacteria samples were subsequently transferred to the agar plates. The T-streaking method was used: each plate was split into quadrants (Quadrants 1, 2, 3, and 4). Beginning in Quadrant 1, a new applicator was dipped into the diluted bacteria sample and streaked over the quadrant in a zigzag pattern. The applicator was discarded and a new one was used to continue the bacteria trail into Quadrant 2. This applicator was discarded and a new applicator was used for Quadrant 3, and so forth. This process was done for every agar plate except the negative controls. A total of 12 applicators were used, where four were used for the plates with lettuce bacteria, four were used for the plates with forearm bacteria, and four were used for the plates with lettuce bacteria that had encountered forearm bacteria. The same applicator was used for the Quadrant 1s of the lettuce bacteria plates, re-dipping into the diluted bacteria sample after each application. Another applicator was used for all the Quadrant 2s, and so forth for each bacteria plate type.

Afterwards, the agar plates were covered and left to sit at room temperature for seven days. A picture of each plate was taken every 24 hours thereafter.

Colony Counting and Analysis

After measuring the growth of the plated bacteria over a span of five days, we calculated the percent of plate area containing bacterial colonies. We used online software to measure the percent of plate area colonized by bacteria. Specifically, we used ImageJ, a Java-based image processing software developed by the National Institutes of Health (NIH) (31). The plates that did not have any bacteria unexposed and exposed to ginger were not analyzed. The picture was uploaded to ImageJ. "Color Threshold" was adjusted so only the areas with bacterial growth were marked in red. The detection of the colonies was not always accurate, so each plate was adjusted differently to estimate the areas of growth best. The percentages of plate covered were measured using the "Analyze Particles" option with a pixel size of zero-infinity, circularity of 0.00-1.00, and edges excluded.

To determine the potential species of the bacteria along with its Gram type, the morphology of the colonies was verified visually using the pictures taken of the plates after five days of growth.

Additional Notes

Due to a lack of bacterial growth in the plates with lettuce bacteria during culturing, the experiment had to be redone. During the preparation for culturing the second time, the same vial of Ultrapure water was reused when re-swabbing the bacteria samples. All the procedures and materials remained the same otherwise. The information in the above sections follows the successful repeat of the experiment and not its initial attempt.

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REFERENCES

1. Doron, Shira, and Sherwood Gorbach. "Bacterial Infections: Overview." *International Encyclopedia of Public Health*, 2008, pp. 196-205. https://doi:10.1016/B978-012373960-5.00596-7
2. Hatfull, Graham F., et al. "Phage Therapy for Antibiotic-Resistant Bacterial Infections." *Annual Review of Medicine*, vol. 73, no. 1, January 2022, pp. 197-221. https://doi:10.1146/annurev-med-080219-122208
3. Morris, Sabrina, and Elizabeth Cerceo. "Trends, Epidemiology, and Management of Multi-Drug Resistant Gram-Negative Bacterial Infections in the Hospitalized Setting." *Antibiotics*, vol. 9, no. 4, April 2020, pp. 196. https://doi:10.3390/antibiotics9040196
4. Munita, Jose M., and Cesar A. Arias. "Mechanisms of Antibiotic Resistance." *Microbiology Spectrum*, vol. 4, no. 2, 8 April 2016. https://doi:10.1128/microbiolspec.VMBF-0016-2015
5. Witte, Wolfgang, et al. "Emergence and spread of antibiotic-resistance Gram-positive bacterial pathogens." *International Journal of Medical Microbiology*, vol. 298, no. 5-6, 1 July 2008, pp. 365-77. https://doi:10.1016/j.ijmm.2007.10.005
6. Eichenberger, Emily, and Joshua Thaden. "Epidemiology and Mechanisms of Resistance of Extensively Drug Resistant Gram-Negative Bacteria." *Antibiotics*, vol. 8, no. 2, 6 April 2019, pp. 37. https://doi:10.3390/antibiotics8020037
7. Friedman, N.D., et al. "The negative impact of antibiotic resistance." *Clinical Microbiology and Infection*, vol. 22, no. 5, May 2016, pp. 416-22. doi: https://10.1016/j.cmi.2015.12.002
8. Pirommas, Techitnutsarut, and Chamchod Farida. "Modeling bacterial resistance to antibiotics: bacterial conjugation and drug effects." *Advances in Continuous and Discrete Models*, vol. 2021, no. 1, December 2021. https://doi:10.1186/s13662-021-03423-8
9. Courvalin, Patrice. "Transfer of Antibiotic Resistance Genes between Gram-Positive and Gram-Negative Bacteria." *Antimicrobial Agents and Chemotherapy*, vol. 38, no. 7, July 1994, pp. 1447-51.
10. Grohmann, Elisabeth, et al. "Conjugative Plasmid Transfer in Gram-Positive Bacteria." *Microbiology and Molecular Biology Reviews*, vol. 67, no. 2, 1 June 2003, pp. 277-301. https://doi:10.1128/MMBR.67.2.277-301.2003
11. Luna-Guevara, J. J., et al. "The Role of Pathogenic *E. coli* in Fresh Vegetables: Behavior, Contamination Factors, and Preventative Measures." *International Journal of Microbiology*, 26 November 2019. https://doi:10.1155/2019/2894328
12. Tuazon, Carmelita U. "Skin and skin structure infections in the patient at risk: Carrier state of staphylococcus aureus." *The American Journal of Medicine*, vol. 76, no. 5, 15 May 1984, pp. 166-71. https://doi:10.1016/0002-9343(84)90260-2
13. Gehad, Youssef, and Mark Springel. "Characterization of antibacterial properties of common spices." *Journal of Emerging Investigators*, vol. 3, no. 1, 3 October 2020, pp. 1-4.
14. Rahmani, Arshad H., et al. "Active ingredients of ginger as potential candidates in the prevention and treatment of diseases via modulation of biological activities." *International Journal of Physiology, Pathophysiology and Pharmacology,* vol. 6, no. 2, 12 Jul. 2014.
15. Abdalla, Wail E. and Abdallah, Emad M. "Antibacterial Activity of Ginger (Zingiber Officinale Rosc.) Rhizome: A Mini Review." *International Journal of Pharmacognosy and Chinese Medicine*, vol. 2, no. 4, 25 June 2018. https://doi:10.23880/ipcm-16000142
16. Akullo, Jolly, et al. "Effect of aqueous and organic solvent extraction on in-vitro antimicrobial activity of two varieties of fresh ginger (Zingiber officinale) and garlic (Allium sativum)." *Heliyon*, vol. 8, no. 9, Sep. 2022. https://doi:10.1016/j.heliyon.2022.e10457
17. Mohamedin, Attiya, et al. "Molecular Effects and Antibacterial Activities of Ginger Extracts against Some Drug Resistant Pathogenic Bacteria." *Egyptian Journal of Botany*, vol. 58, no. 1, Apr. 2018. https://doi:10.21608/ejbo.2018.1090.1100
18. Zhang, Lingling, et al. "Antibacterial activity and mechanism of ginger extract against Ralstonia solanacearum." *Journal of Applied Microbiology*, vol. 133, no. 4, 1 Oct. 2022. https://doi:10.1111/jam.15733
19. Rahman, Mijanur, et al. "In vitro evaluation of probiotic and bacteriocinogenic potentiality of Lactobacillus plantarum and Lactobacillus delbrueckii isolated from vegetables in Chittagong region, Bangladesh." *Malaysian Journal of Microbiology*, vol. 15, no. 2, Apr. 2019. https://doi:10.21161/mjm.180070
20. Hossain, MS, et al. "Identification and Antibiogram Assay of Escherichia Coli Isolated From Chicken Eggs." *Journal of Bio-Science*, vol. 29, no. 1, June 2021. https://doi:10.3329/jbs.v29i0.54828
21. Kelly, B. G., et al. "Gene transfer events and their occurrence in selected environments." *Food and Chemical Toxicology*, vol. 47, no. 5, May 2009, pp. 978-983. https://doi:10.1016/j.fct.2008.06.012
22. Tanaka, Tomohiro, et al. "A Hidden Pitfall in the Preparation of Agar Media Undermines Microorganism Cultivability." *Applied and Environmental Microbiology*, vol. 80, no. 24, December 2014, pp. 7659-7666. https://doi:10.1128/AEM.02741-14
23. Kell, Douglas B., and Louise C. Kenny. "A Dormant Microbial Component in the Development of Preeclampsia." *Frontiers in Medicine*, vol. 3, no. 60. 29 November 2016. https://doi:10.3389/fmed.2016.00060
24. Nocera, Francesca Paola, et al. "On Gram-Positive- and Gram-Negative-Bacteria-Associated Canine and Feline Skin Infections: A 4-Year Retrospective Study of the University Veterinary Microbiology Diagnostic Laboratory of Naples, Italy." *Animals*, vol. 11, no. 6, June 2021. https://doi:10.3390/ani11061603
25. Smoglica, Camilla, et al. "Antibiotic-Resistant Bacteria Dissemination in the Wildlife, Livestock, and Water of Maiella National Park, Italy." *Animals*, vol. 13, no. 3, 27 January 2023. https://doi:10.3390/ani13030432
26. Manyi-Loh, Christy, et al. "Antibiotic Use in Agriculture and Its Consequential Resistance in Environmental Sources: Potential Public Health Implications." *Molecules*, vol. 23, no. 4, April 2018. https://doi:10.3390/molecules23040795
27. Donskey, Curtis J. "Antibiotic regimens and intestinal colonization with antibiotic-resistant Gram-negative bacilli." *Clinical Infectious Diseases*, vol. 43, no. 2,

https://doi.org/10.59720/23-123

September 2006, pp. 62-69. https://doi:10.1086/504481
28. Manohar, Prasanth, et al. "Transfer of Antibiotic Resistance Genes from Gram-positive Bacterium to Gram-negative Bacterium." *bioRxiv*, 2 November 2020. https://doi:10.1101/2020.11.01.364331
29. Scäfer, A., et al. "High-frequency conjugal plasmid transfer from Gram-negative Escherichia coli to various Gram-positive coryneform bacteria." *Journal of Bacteriology*, vol. 172, no. 3, March 1990, pp. 1663-1666. https://doi:10.1128/jb.172.3.1663-1666.1990
30. Breijyeh, Zeinab, et al. "Resistance of Gram-Negative Bacteria to Current Antibacterial Agents and Approaches to Resolve It." *Molecules*, vol. 25, no. 6, March 2020. https://doi:10.3390/molecules25061340
31. "ImageJ." U.S. *National Institutes of Health*. imagej.nih.gov/ij/. Accessed Aug. 2022.