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Article

Characterizing the evolution of antibiotic resistance in commercial *Lactobacillus* strains

Ethan Gibbs, Jennifer Gibbs
Olentangy High School, Lewis Center, Ohio

SUMMARY

Throughout the world, millions of patients consume probiotic supplements and fermented foods for their perceived health benefits. Recent research suggests that the ingestion of bacteria commonly associated with probiotics negatively correlates with the prevalence of health conditions such as antibiotic-associated dysbiosis and can benefit immunocompromised patients through competitive inhibition. Therefore, healthcare providers often recommend co-administration of probiotics and antibiotics during treatment. Despite the benefits conferred by co-administration, the transfer of antibiotic-resistance genes from probiotics to pathogenic bacteria residing in the gut microbiome remains a significant risk to patient health, as many species are known to be resistant. We sought to determine how antibiotic resistance and its transferability developed among a commonly consumed species of probiotic, *Lactobacillus*. We hypothesized that these bacteria would demonstrate increased resistance to an antibiotic when administered over successive generations, that resistance would specifically develop during the first two generations of antibiotic exposure, and that antibiotic resistance could be modeled to predict future behavior in response to other antibiotics. We determined the transferability of antibiotic resistance by exposing *Escherichia coli* to resistant Lactobacillus and testing for antibiotic resistance. Our results demonstrated that *Lactobacillus* develops antibiotic resistance logarithmically with each generation and variably depending on the class of the antibiotic administered. Further, we created a model to predict how antibiotic resistance developed. Our findings may influence how healthcare providers evaluate the risks associated with probiotic co-administration in immunocompromised patients.

INTRODUCTION

The human microbiome, found throughout the gastrointestinal system, is home to many microorganisms, including bacteria, archaea, viruses, fungi, and protozoa, and its composition can profoundly affect human health and subsequent disease (1). Commensal (neutral) microorganisms within the microbiome of healthy humans eliminate opportunities for pathogens (harmful bacteria) to proliferate by outcompeting them for resources. Further, they produce a multitude of vitamins that are beneficial to the human body (1). However, ingestion of large amounts of pathogenic bacteria (e.g., food poisoning) destabilizes microbiome composition and results in various clinical symptoms, including diarrhea and nausea (1). Antibiotics can similarly destabilize the gut microbiome; however, their effect is more profound. Even a brief course of antibiotic therapy can create disturbances that last for up to four years (2). In a recent study, Shaw et al. explained this phenomenon by characterizing the human microbiome as a stability landscape; its composition is set at equilibrium and may shift due to environmental factors, such as antibiotic treatment (3). Explaining this shift requires an understanding of how antibiotics work.

Antibiotics act by targeting specific molecular mechanisms shared by most bacterial cells. While antibiotics may be prescribed to eliminate pathogenic bacteria, they affect many commensal microbes collaterally through analogous mechanisms. The intensity of this effect depends on the antibiotic(s) used. Antibiotic-induced disturbances may present in areas outside the gut as well. Recent research suggests that reductions in commensal microbes can impact systemic immunity (4). Further, broad-spectrum antibiotics affect far more than the pathogens targeted by therapy, leading to major ecological imbalances. Health conditions such as irritable bowel disease, antibiotic-associated diarrhea, metabolic syndrome, and obesity have been linked to these imbalances (4).

The severity of these health conditions ultimately depends on the drug potency and duration of use, amount of inoculum ingested, and host factors (e.g., biological predisposition) (5). For example, pseudomembranous colitis is commonly caused by the pathogen, *Clostridium difficile,* following antibiotic therapy and is one of the most researched conditions involving the gut microbiome (6). Despite ongoing research efforts, our knowledge of antibiotics and their effects on human systems remains limited. Evaluating antibiotic use and the long-term implications resulting from treatment is becoming increasingly relevant (7).

Many patients and consumers have turned to commercial products containing doses of the beneficial bacteria to combat the symptoms of microbial dysbiosis, which results from an imbalance of bacteria in the gut. Increasing evidence indicates that probiotics may confer a multitude of benefits upon their host, and their appearance in foods and dietary supplements is on the rise as a result (8). In the 2012 National Health Interview Survey, four million adults in the United States had used probiotics within the past month (9). Notably, probiotics were one of the most used dietary supplements, following vitamins and minerals, and their use has increased four-fold since 2007 (9).

Co-administering probiotics during antibiotic therapy

is common practice and may protect the microbiome from antibiotic-induced disturbances through competitive exclusion, reinforcement of the gut barrier, and enhancement of immune function (5). In patients treated with antibiotics, probiotics restore the commensal microbiota and decrease sensitivity to future disturbances. Clinical evidence suggests that probiotic administration is associated with reductions in the prevalence of antibiotic-associated diarrhea (10). Additionally, probiotics reduce the risk of antibiotic-induced infections associated with dysbiosis. The administration of *Lactobacillus rhamnosus* has reportedly reduced the risk of yeast infection and bacterial vaginosis (11). Therefore, healthcare providers value probiotics as supplements to antibiotics, and further research to determine their dosage and duration of treatment would be valuable (11).

Despite the potential benefits, concerns exist surrounding the safety of probiotic use and the consumption of foods containing probiotic bacteria. The transmission of bacteria through the wall of the intestines and the release of toxic molecules remains areas of concern (8). For example, bacteria such as *L. rhamnosus* and *L. casei* increase the risk of bacteremia (bloodborne bacteria) in immunocompromised patients despite improved safety profiles of probiotics (11). Many other lactic acid bacteria, including *L. plantarum* and *L. acidophilus*, have been associated with bacteremia and endocarditis (bacteria attacking the heart). Lastly, and most importantly, there is potential for the transfer of antibiotic resistance in these bacteria (12). Many species of *Lactobacillus* have been found to contain plasmids, or pieces of genetic material that move from one bacterium to another, with antibiotic-resistance genes. These plasmids have the potential to transfer antibiotic resistance to harmful pathogens in the gut (12).

Given the safety concerns surrounding probiotics and the increasing number of probiotic consumers, more research on antibiotic-resistant probiotics is needed to help patients make informed decisions. A study investigating the influence of antibiotics on resistance genes could enhance public understanding of the dangers of probiotic bacteria (5). As antibiotic-resistant pathogens are becoming increasingly abundant in healthcare and community environments, new antibiotics are needed to fight these bacterial threats. Antibiotics take significantly longer to develop than the time it takes for new resistant pathogens to evolve (13). Thus, it is becoming increasingly relevant to devise strategies for controlling and preventing the spread of resistance. Knowledge about the functions of probiotic bacteria is limited, and a better understanding of how probiotics behave during antibiotic therapy will help optimize treatment (14).

The objective of our study was to determine how antibiotic resistance evolves during co-administration. We hypothesized that the bacteria tested would demonstrate increased resistance to each antibiotic administered during successive generations, the acquisition of resistance would occur during the first two generations of antibiotic exposure, and we could create a model to predict future behavior.

Despite considering multiple lactic acid bacteria and probiotics, evidence concerning *Lactobacillus* led us to include it as the primary subject bacteria. Studies suggest that subspecies belonging to this genus effectively combat antibiotic-associated diarrhea and are more likely to be co-administered with antibiotics (17). Our results indicated that *Lactobacillus* develops antibiotic resistance logarithmically with each generation and variably depending on the antibiotic class administered. Based on these results, we were able to create a model to predict how antibiotic resistance will develop. Our findings may influence how healthcare providers evaluate the risks associated with probiotic co-administration in immunocompromised patients.

RESULTS

We isolated and inoculated bacteria from a random commercial probiotic into MRS (Man, Rogosa, and Sharpe) growth broth, recollected the sample, and performed a Gram stain (**Figure 1**). We classified the bacteria as *Lactobacillus* considering their phenotypic similarities (Gram-positive, rod-shaped) and the presence of sodium acetate in the MRS broth, which inhibits competing bacteria. We plated bacteria in separate dishes and exposed each dish to a different set of antibiotics that are frequently used in clinical settings. We then measured the inhibitory zones, which we interpreted as the degree to which the bacteria were sensitive to each antibiotic; we speculated that bacteria closest to the antibiotic disk after incubation had the highest amount of resistance and exposure time. From each dish, we re-plated bacteria from the edge of the inhibitory zone and exposed them again to the same set of antibiotics, which we denoted as consecutive exposure. Finally, we measured the inhibitory zone after each exposure and compared it to the previous generation of bacteria.

We originally hypothesized that consecutive exposure to antibiotics would result in the development of antibiotic-resistant probiotic bacteria. The quantitative results of our study supported this hypothesis, as consecutive generations appeared to grow within a smaller range of the antibiotic disks of the same concentration. For example, the first and second generations of *Lactobacillus* exposed to tetracycline displayed a visible decrease in inhibitory zone diameter after a single, consecutive exposure (**Figure 2**). We observed similar results with streptomycin and erythromycin treatment.

The data we collected from the three subject trials showed that, on average, resistance most frequently developed after the first and second generations of antibiotic exposure (**Table 1**). Across all three antibiotic classes, the development of resistance displayed a logarithmic trend, such that each consecutive generation exhibited a smaller change to antibiotic susceptibility. Further, we plotted the experimental

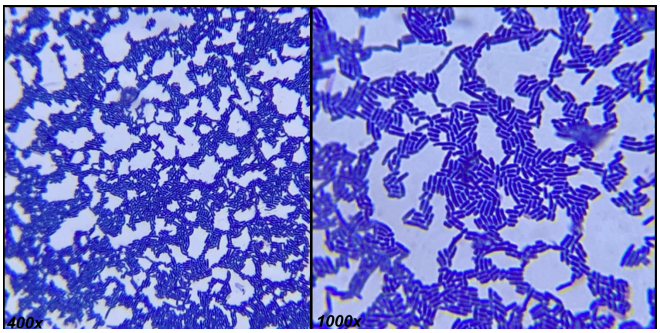

Figure 1: Bacteria isolated from a commercial probiotic share characteristics with *Lactobacillus*. Images were obtained with 400x (left) and 1000x (right) magnification. *Lactobacillus* are Gram-positive (stain as blue/violet) and rod-shaped bacteria.

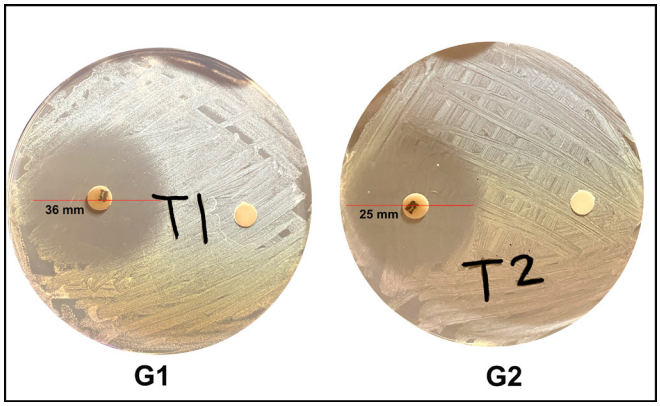

Figure 2: Increased resistance to tetracycline after consecutive exposures. Images of the first and second generations of bacteria exposed to a tetracycline-releasing disk (on the left side of each dish, labeled "TE30"). "T" indicates the generation of the specific antibiotic used, and "G" indicates the overall generation of the bacteria. Bacterial colonies from the edge of the inhibitory zone in "T1" (N = 1) were plated on "T2" (N = 1) and exposed to an identical tetracycline antibiotic disk. The horizontal red lines demarcate the diameter of the inhibitory zone surrounding the antibiotic-releasing disk. The yellow disks on the right of the plates are negative controls, which have no associated inhibitory zones.

data graphically and fit each graph to logarithmic curves. The coefficient of determination for each regression averaged greater than 0.99, suggesting a high correlation to the observed data (**Figure 3**).

Compared to the experimental results, predictions that we made using the generational average regression displayed a significant degree of error. This indicated that, across all antibiotics, the development of resistance was dependent on the initial zone diameter and initial change. Therefore, we speculated that to make more accurate predictions, the initial zone diameter (G1) and initial change (G1 to G2) should be experimentally obtained. However, changes to the initial change in sensitivity in consecutive generations can be modeled, as every natural logarithm approaches the same limit.

To confirm this, we calculated hypothetical values for generations three and four to connect this model to the experimental results (**Table 2**). To determine the model's

Table 1: Average zone diameter (mm) of antibiotics tested for each generation of *Lactobacillus*. "G#" indicates generation number and "Sens." Indicates sensitivity (Sensitive or Resistant) based on the interpretive cut-off of ≤14 mm. Sample size of (N = 1) for each antibiotic trial. Negative controls (sterile paper disks) were expected to have an inhibitory zone of zero millimeters.

Antibiotic (µg/disk)	G1 (mm)	Sens.	G2 (mm)	Sens.	G3 (mm)	Sens.	G4 (mm)	Sens.
Tetracycline (30 µg/disk)	36	S	25	S	16	S	13	R
Streptomycin (10 µg/disk)	20	S	15	S	13	R	11	R
Erythromycin (15 µg/disk)	38	S	24	S	16	S	14	R
Generation Average	31.33	S	21.33	S	15.00	S	12.00	R
Negative Control	0	N/A	0	N/A	0	N/A	0	N/A

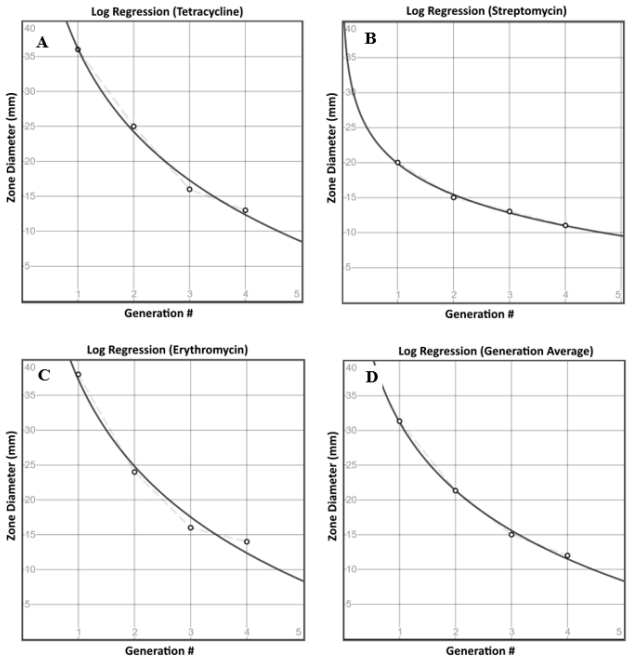

Figure 3: Regressions indicate high correlation to the natural logarithm. Generation of exposure is plotted on the X axis, and zone diameter (mm) is plotted on the Y axis. (A) Tetracycline, R^2 = 0.9917, (B) Streptomycin, R^2 = 0.9949, (C) Erythromycin, R^2 = 0.9825 over four generations. (D) Averages for each generation were calculated and fit R^2 = 0.9972 to a fourth curve. The *R*-squared value indicates high correlation to data. Sample size of (N = 1) for each antibiotic trial.

efficacy, we calculated the average percent error by comparing the hypothetical values to the experimental data (**Table 1**). We were able to predict zone diameters within an average of ten percent of the experimental values. The model was also able to accurately predict the sensitivity status of all but one trial (Tetracycline, G4).

We also randomly sampled, Gram stained, and observed bacteria from the edge of the inhibitory zone of the fourth generation of each antibiotic trial. No significant changes to bacterial morphology (the size, shape, or structure) of the streptomycin and erythromycin groups were observed. However, larger variants were prominent in the Gram stain of generation "T4" (**Figure 4**).

Finally, to indicate the presence of plasmids, we inoculated bacteria from the fourth generation of each antibiotic in a separate tube of MRS broth. When sufficient turbidity was visible, we siphoned one milliliter of broth containing bacteria (from each trial) into a distinct broth in which *E. coli* could also be cultured. Afterward, we isolated a single colony of *E. coli* and swabbed it onto a plate containing each antibiotic disk and negative control. We recorded the results of the single-trial disk-diffusion test (**Table 3, Figure 5**). The *E. coli* appeared to exhibit above-average sensitivity to the effective concentrations of streptomycin and erythromycin, but resistance to tetracycline.

DISCUSSION

Our study investigated the effect of consecutive antibiotic exposures on the development of intrinsic and transferrable resistance in a commercial probiotic strain. Our results

Table 2: Predicting changes in sensitivity. The '*' indicates that the values for this generation were not experimentally obtained. Sample size of (N = 1) for each antibiotic trial.

Antibiotic	G1	Sens.	G2	Sens.	G3*	Sens.	G4*	Sens.
Tetracycline	36	S	25	S	19.5	S	15.8	S
Streptomycin	20	S	15	S	12.5	R	10.8	R
Erythromycin	38	S	24	S	17	S	12.3	R

supported the proposed hypotheses and two auxiliary findings. We concluded that *Lactobacillus* developed resistance in response to each antibiotic that approximated a logarithmic curve and that this curve could predict future behavior with some degree of error (**Figure 3**). When considering the biological significance of the predictive model, however, the calculated error may be negligible. Since the zone diameters used in this study (and other clinical laboratories) represent a generalized sensitivity, predicting when resistance will develop is more relevant to healthcare providers than a precise measurement (15). The generation at which resistance developed was accurately recorded for all but one generation, however, this discrepancy can be explained by the lenient nature of the interpretive standards (**Table 2**).

In addition to changes in resistance among *Lactobacillus*, we found that the morphology of tetracycline-exposed bacteria changed compared to the first generation. This suggests the presence of structural mutations in response to tetracycline exposure or multiple, morphologically different strains of *Lactobacillus*. There may also be a connection between the morphology of these bacteria and their transferability of resistance (18). The bacteria became darker, larger, and longer (**Figure 4**). We did not observe this change in morphology in the streptomycin and erythromycin antibiotic trials. However, the change in appearance likely resulted following consecutive tetracycline exposure because the bacteria were isolated from a single colony, and the morphology was not observed in previous generations. This could be addressed in an additional experiment, where only tetracycline is utilized.

Since tetracycline-exposed bacteria were the only subjects to express these qualitative changes, we also considered their morphology in the context of the transferable resistance genes. *E. coli* sampled from the growth medium containing

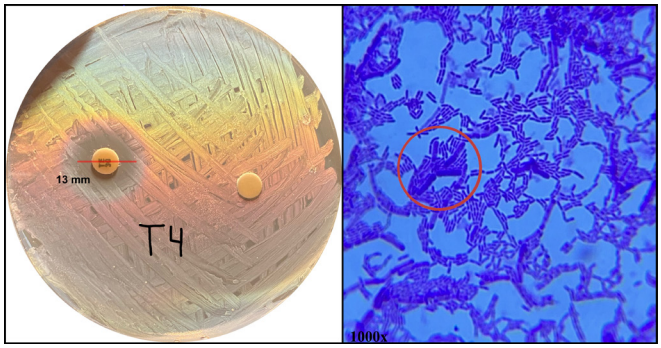

Figure 4: Changes in bacterial morphology observed in tetracycline-exposed bacteria. Agar plate (left) and image of Gram stained, fourth generation bacteria exposed to tetracycline (right). Partially resistant colonies of *Lactobacillus* along the inhibitory zone were isolated. The original sample (Figure 1) did not contain larger variants (shown within the red circle).

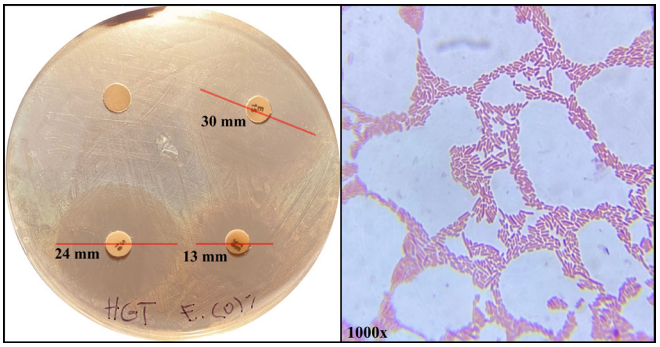

Figure 5: Resistance to tetracycline observed in *E. coli*. Agar plate (left) and Gram stain (right) of the single trial disk-diffusion test of *E. coli*. The Gram-negative bacteria are notable for their carriage of tetracycline plasmids (23). Inhibitory zones were measured for different antibiotics, including (clockwise) erythromycin (30 mm), tetracycline (13 mm), streptomycin (24 mm), and negative control (0 mm).

Lactobacillus were resistant to tetracycline, suggesting that cell wall differences could factor into how they respond to the antibiotic (**Figure 5**). Although a relationship between bacterial morphology and the development of resistance plasmids remains plausible, examining this phenomenon was beyond the scope of our study.

Addressing the transferability of resistance genes themselves is essential, as the non-pathogenic *E. coli* used in this study is phenotypically similar to other Gram-negative pathogenic bacteria that inhabit the human microbiome. Thus, patients risk transferring these genes from antibiotic resistance reservoirs to other commensal and pathogenic species. We speculated that patients face a higher risk of developing antibiotic resistance because the scale of the microbiome allows multiple genetic conjugations to coincide. Based on our results, certain antibiotics may be more susceptible to resistant plasmids. Only tetracycline-exposed bacteria were identified as carriers of plasmids in our study, an example of horizontal gene transfer (16). In a clinical setting, variability implies that the optimization of antibiotic and probiotic co-administrative therapy requires medical specialists to be aware of the antibiotics for which resistance genes are frequently found

Table 3: Zone diameter (mm) of antibiotics from the single trial disk-diffusion test of *E. coli*. Presence of transferable resistance in the fourth generation is indicated by a zone diameter of ≤15 mm. Pure *Escherichia coli* was incubated in a mixture of the T4, S4, and E4 samples then sub-cultured before the data in this table was collected. Sample size of (N = 1) for each antibiotic trial.

Antibiotic (µg/disk)	G4/*E. coli* (mm)	Sens.
Tetracycline (30 µg/disk)	13.00	R
Streptomycin (10 µg/disk)	24.00	S
Erythromycin (15µg/disk)	30.00	S

within bacterial plasmids.

The logarithmic trends we observed suggest that bacteria living within patients given adjunct probiotics will develop resistant variants in fewer than five consecutive doses. However, the small number of bacteria species evaluated in this study, relative to the microbiome of the gut, confounds the interpretation of this finding. In a more diverse and densely populated environment with uncontrolled factors, the transfer of genetic material could accelerate the development of antibiotic resistance. Thus, antibiotic treatment should be optimized to completely kill all bacteria before re-population to avoid the risk of resistance genes pooling among commensal bacteria.

Additionally, several sources of error in the experimental process could have influenced the results. Firstly, there was a lack of control over air contaminants due to the inaccessibility of laminar flow in the lab environment. The selectivity of the MRS medium and *Lactobacillus* likely mitigated contamination in the first series of experiments. However, the non-selective agar used to test transferable resistance may have led to contaminated *E. coli* plates. We observed no contaminants despite performing microscopic observations before and after the procedure, but any missed contaminant bacteria may have affected data interpretation.

Secondly, there was the potential for error and bias during data collection and analysis. Due to time and financial restrictions, we could not complete multiple trials for each antibiotic to provide more replicable results. While the changes between most generations were large and thus likely surpassed deviation, this could have had an impact on the specificity of our results, as there was a potential for samples with greater innate resistance. We speculated that additional trials would result in average diameters for each generation that more closely correlate with the predictive model.

Several revisions to our experiments may improve the clarity of future results obtained. As previously discussed, our procedure lacked multiple trials for each antibiotic class. However, we could also expand the experiment to include the use of more antibiotics, probiotics, and environmental conditions. The conclusions within our study are strictly limited to commercial *Lactobacillus* of undefined subspecies. Additionally, it is important to consider that the *E. coli* used could have been innately resistant to tetracycline before the study was conducted. We could have addressed this by testing the susceptibility of the bacteria before inoculating it with the subject *Lactobacillus*. Lastly, we could improve upon the accuracy of claims regarding genetic information in our study by implementing genetic sequencing technology. The presence of plasmids and intrinsic resistance genes could be directly confirmed through data rather than inferences made based on observations, as seen in this study.

Our results are valuable to probiotic co-administration patients. Depending on the probiotics and antibiotics consumed, patients may be at higher risk of developing reservoirs of transferable antibiotic resistance in their microbiomes. In the context of the antibiotic resistance epidemic, patients should be mindful of the effects of antibiotic treatment on their microbiomes. Our study will help individuals and medical specialists make informed decisions about co-administration. It may also provide a method of evaluating the risks associated with probiotic co-administration in immunocompromised patients.

Furthermore, the development of multi-drug resistant probiotic therapies remains a crucial avenue that researchers should explore. Certain bacteria, such as *Lactobacillus*, may be rendered resistant to multiple antibiotics to minimize the destructive effects of antibiotics and potentially antibiotic-resistant pathogens (19). Quantifying the response of probiotic bacteria to antibiotics may be critical to the development of these alternative therapies.

MATERIALS AND METHODS

Isolating and Classifying the Strain

Samples of pure *Lactobacillus* were obtained from a commercially available probiotic (Inessa) supplement claiming to contain *L. rhamnosus* and inoculated in a tube containing 10 mL of De Man Rogosa Sharpe (MRS) broth (HiMedia). The design of this medium permits large quantities of *Lactobacillus* to be cultured, as it contains the amino acids and vitamins required by *Lactobacillus*. Since many commercial products contain strains other than those listed on the label, the specific strain of *Lactobacillus* obtained remained ambiguous (20).

After incubating the samples for 24 hours at 37°C until visibly turbid, we collected bacteria by dipping a sterile swab into the growth medium and rotating it against the side to remove excess fluid. The bacteria were then streaked onto a plate containing 15 mL of MRS agar (HiMedia) and incubated again for 24 hours at 37°C. Finally, a single colony was isolated using a sterile inoculating loop and transferred to a separate tube containing 10 mL of MRS liquid growth medium.

The bacteria from the isolated colony were classified using traditional Gram staining. The procedure stained the bacterial cell wall such that Gram-positive strains appeared violet and Gram-negative strains appeared pink, allowing for accurate classification (*Lactobacillus spp.* are Gram-positive) and detecting contaminant Gram-negative bacteria. To view the stained bacteria under 1000x magnification, immersion oil microscopy was used. Immersion oil increases the resolution of the image produced while maintaining high magnification.

Preparation of Agar Plates

Bacteria from the isolated colony were collected from the tube by dipping a sterile swab into the medium, then rotating it against the side of the tube to remove excess fluid. The swab containing the bacteria was then streaked onto an agar plate containing 15 mL of MRS agar. To ensure even distribution, bacteria were swabbed over the surface of the agar three times, rotating sixty degrees each time. This procedure, which creates a lawn culture, was repeated for each trial and each generation of bacteria throughout the study.

Antibiotic susceptibility testing disks (6 mm diameter) for tetracycline (30 µg), erythromycin (15 µg), and streptomycin (10 µg) were placed on the left side of each plate (Carolina Biological). Negative control disks (Carolina Biological) made of sterile paper were placed on the right side of each plate. The disks were placed in the center of their respective section and pressed lightly to ensure contact with the agar. The agar plates were then flipped upside down to avoid condensation and incubated at 37 °C for 24 hours. For the preceding methods, the Kirby Bauer Disk Diffusion Susceptibility Test Protocol was referenced (21).

Measuring and Interpreting Inhibitory Zones

Data were collected by measuring the diameter (mm) of

the circular zone surrounding the antibiotic disk where no bacteria grew, which we referred to as the "inhibitory zone". If the edge of one side of the inhibitory zone was obscured by the edge of the plate and thus could not be measured, the distance from the center of the antibiotic disk to the edge of the zone in the opposite direction was measured and doubled to determine the diameter. Additionally, when the inhibitory zone was impacted by partially resistant bacteria, the radius was measured from the closest visible edge to the center and multiplied by two. Partially resistant bacteria that exhibited reduced colony density in the presence of the antibiotic disk were interpreted as viable. Lastly, bacteria were considered to display "total resistance" when the inhibitory zone diameter was measured to be the diameter of the disk (6 mm).

Since the isolation of bacteria from probiotic products occurs infrequently, interpretive criteria created by Temmerman *et al*. were used to assess the susceptibility of *Lactobacillus* subspecies on MRS agar (22). The bacteria were considered resistant if the inhibitory zones were ≤14 mm for tetracycline and ≤13 mm for erythromycin. Temmerman *et al*. suggested ≤13 mm as the cut-off value for kanamycin; therefore, this value was chosen for streptomycin since the two drugs belong to the same antibiotic class (22). Based on these interpretive criteria, the cut-off for the resistance of the *Lactobacillus* used in this study was approximated to be ≤14 mm.

Simulating Generational Exposure

To determine how *Lactobacillus* develops intrinsic and transferable resistance during a course of antibiotics, a method for simulating repeated, non-lethal exposures was developed. It was speculated that bacteria closest to the antibiotic disk after incubation had the highest amount of intrinsic resistance and exposure time. Thus, consecutive generations were created by collecting the bacteria on the edge of the inhibitory zone with an inoculating loop and transferring them into a separate tube of MRS liquid medium. Following the same procedure, a new generation was derived from the previous one after each disk-diffusion trial.

Proof of Transferable Resistance

At the end of each antibiotic trial, the final generation of *Lactobacillus* was inoculated in tryptic soy broth with pure *E. coli*, a gram-negative bacteria species. This broth was selected because it best supported the growth of *E. coli*. After incubating at 37°C for 24 hours, the mix of bacteria was collected from the tube using a sterile swab and streaked onto a plate containing 15 milliliters of Mueller Hinton Agar (MHA). The MHA is suitable for non-fastidious bacteria and is clinically supported for antibiotic susceptibility testing (21). Next, a single colony of *E. coli* was sub-cultured and plated with each antibiotic disk and a blank control and incubated at 37 °C for 24 hours. If the resistance present in the subject *Lactobacillus* was carried on mobile genetic elements, it would also be present in the inoculated *E. coli* after growing in the same medium due to horizontal gene transfer (23). The data from the susceptibility test was interpreted based on the Clinical Laboratory Standards Institute quality control limits using MHA (24) and the ASM zone diameter interpretive standards for *E. coli* (21). Interpretive standards may vary slightly by source, but the cut-off for the resistance of the *E. coli* used in this study was approximated to be ≤15 millimeters. This test was intended to support the transferability of resistance in the *Lactobacillus* samples used throughout this study.

Data Analysis

The data from this study were analyzed for biological significance by comparing antibiotic zone diameters to their interpretive standards and calculating the rate of change between generations. The Desmos Graphing Calculator was used to approximate a logarithmic regression curve for each antibiotic using the formula (Equation 1). The regression equation for the generational averages was derived using the Desmos Graphing Calculator to create a mathematical model (Equation 2).

Equation 1: Regression Equations

Equation 2: Rates of Natural Logarithms

$$t(x) = 36.11 - 17.14 \ln(x),\ t'(x) = -\frac{17.14}{x}\ mm/gen$$

$$s(x) = 19.84 - 6.41 \ln(x),\ s'(x) = -\frac{6.41}{x}\ mm/gen$$

$$e(x) = 37.27 - 17.96 \ln(x),\ e'(x) = -\frac{17.96}{x}\ mm/gen$$

$$g(x) \sim 31.19 - 14.12 \ln(x),\quad g'(x) \sim -\frac{14.12}{x}\ mm/gen$$

Note. These percentages reflect a fraction of the previous

$$-\frac{14.12}{2} = -7.06 \qquad -\frac{14.12}{3} = -4.71 \qquad -\frac{14.12}{4} = -3.53 \qquad -\frac{14.12}{5} = -2.82$$

$$\frac{7.06}{14.12} \sim 50\% \qquad \frac{4.71}{7.06} \sim 67\ \% \qquad \frac{3.53}{4.71} \sim 75\% \qquad \frac{2.82}{3.53} \sim 80\%$$

rate of change. For example, if the change in sensitivity from generation one to generation two was ten millimeters, the change from generation two to generation three is expected to be five millimeters.

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