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RESEARCH ARTICLE

Environmental Isolate Developing Antibiotic Resistance by Complementation

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ABSTRACT

Antibiotic resistance is a growing concern within the scientific community. With few new antibiotics being introduced and an increasing number of resistant microbes, routine bacterial infections are becoming more difficult to treat in clinics and hospitals. The purpose of this study is to compare the ability of two environmental isolates – *Staphylococcus aureus* (*S. aureus*) and *Exiguobacterium undae* (*E. undae*) to grow in solutions of increasing concentrations of tetracycline and ciprofloxacin. After the bacteria showed grow in the solutions, antibiotic susceptibility was tested by examining zones of inhibition on Trypticase Soy Agar (TSA) plates. Our results indicate both isolates were initially susceptible to each antibiotic. The isolates were grown individually and mixed to determine if the isolates could gain resistance to the antibiotics in either environment. Our results demonstrate that *E. undae* could grow and become resistant in mixed cultures when grown in the presence of *S. aureus* reflecting the ability of *S. aureus* to complement microbial growth. Along with the ability of *S. aureus* to complement the growth of *E. undae*, it was also able to develop resistance to both ciprofloxacin and tetracycline through repetitive exposure.

Keywords: Antibiotic resistance, *Staphylococcus aureus*, *Exibuobacterium undae*, tetracycline, ciprofloxacin, complementation

Introducution

Bacteria are challenging scientists as they become increasingly resistant to antibiotics. Infections caused by resistant bacteria have several negative consequences on their host that extend beyond the classic symptoms associated with infection. Treatment of disease is often delayed if the initial antibiotic chosen is not effective due to resistance. Individuals also experience expensive and prolonged hospital visits, additional antibiotic therapy expenses, and restricted, toxic therapy options[1]. While antibiotics use a variety of mechanisms, bacteria are continually evolving to resist these mechanisms. With antibiotic resistance on the rise, anti-virulence factors are also becoming more common when trying to treat infections. Anti-virulence factors will not kill bacteria, but they do allow immune systems to aid in fighting off infections. In particular, a study published in *PLoS* biology found that when the correct combination of anti-virulence factors are chosen they can work synergistically with antibiotics to clear infections[2]. Two other research groups demonstrated synergy between oxacillin and phenothiazines and their effect in reducing the resistance of *Staphylococcus aureus* (*S. aureus*) to oxacillin[3]. Antibiotic effectiveness has also been enhanced by the use of antibiotics and bacteriophages against food borne microbes as measured in the laboratory by disc diffusion assays[4].

The aim of this study is to explore the question of whether or not an environmental isolate – *Exiguobacterium undae* (*E. undae*) could grow or even develop resistance to a pair of antibiotics – tetracycline and ciprofloxacin individually and when grown as a mixed culture with a laboratory strain of *Staphylococcus aureus* (*S. aureus*). *S. aureus* is a Gram-positive cream or yellow bacterium found in soil, water and in humans as part of the normal flora on mucous membranes. Some strains of *S. aureus* are pathogenic and can produce biofilms to support general microbial growth[5]. Wound samples taken from a hospital in Nepal studied the affect biofilms had on antibiotic resistance in *S. aureus.* Out of the 76 samples of *S. aureus* isolated, 35 were biofilm producers. The biofilm producers were found to have a higher rate of multidrug resistance. Also out of those 76 *S. aureus* samples, 98.7 % of them were susceptible to tetracycline and 72.4 % were resistant to ciprofloxacin[6]. *E. undae* is also a Gram-positive soil bacterium that is non-pathogenic[7] and produces an orange-colored pigment. A study focused on the environmental effects of antibiotic resistance in microbes detailed that the increasing amount of antibiotic and heavy metal resistance bacteria cause disruptions of the ecological functions that organism inhabits. These environmental bacteria can often be found to exist in biofilms, especially in bodies of water or wastewater treatment plants. The bacteria found in these biofilms were noted to have a higher amount of antibiotic resistance genes than bacteria outside of the biofilm[8].

Tetracycline is a broad-spectrum antibiotic that works to inhibit protein synthesis by preventing attachment of aminoacyl-tRNAs to the ribosomal A site. This antibiotic is unfortunately becoming less effective today as bacteria are developing ribosomal protection proteins or efflux pumps as resistance mechanisms[9]. Ciprofloxacin is a fluoroquinolone drug and also a broad-spectrum antibiotic that inhibits the activity of DNA gyrase enzymes necessary to maintain and adapt DNA supercoiling structures. Ciprofloxacin is often used on Gram-negative bacteria and

can also be used on mixed infections. As with tetracycline, bacteria are becoming resistant to ciprofloxacin by the use of efflux pump and mutations in DNA gyrase enzymes[10]. This experiment was set up to test the ability of *S. aureus* and *E. undae* to grow in increasing concentrations of tetracycline and ciprofloxacin individually to the point of becoming resistant and in a resistant mixed culture as well.

Materials and Methods

A water sample was collected at 45°08'30.6"N 94°30'08.2"W near Litchfield, Minnesota. Samples were diluted (50:200) with sterile H_2O and plated on Dilution Broth (DB - 6 gm tryptone, 9 gm Bacto agar in 650 ml of distilled H_2O) plates. After two to three days growth in a room temperature incubator, colonies (including *E. undae*) were isolation streaked on DB plates and re-incubated until isolated by visual inspection. At this point the *S. aureus* and *E. undae* isolates were moved onto Trypticase Soy Agar (TSA) plates until the experiments could begin. Initial susceptibility and final resistance of each isolate (and the mixed culture) to both antibiotics was determined by measuring zones of inhibition using Becton Dickinson BD BBL Sensi Discs antibiotic discs on TSA plates. The growth of isolates either alone or as mixed colonies was done in 10 ml Trypticase Soy Broth (TSB) tubes that were placed in a New Brunswick Scientific C25 Incubator-Shaker set at 27°C and 150 revolutions per minute. Powder forms of both antibiotics were from Sigma Aldrich (catalog T7660 for tetracycline and 17850 for ciprofloxacin) and dissolved in sterile H_2O as 1 mg/ml (ciprofloxacin) and 10 mg/ml (tetracycline) solutions. Growth was monitored by absorbance at 600 nm using a Beckman DU-640 UV/Visible spectrophotometer. Between rounds of increasing concentrations of antibiotics inoculation loops were used to create isolation streak plates from the 10 ml TSB tubes and incubated at room temperature.

The *E. undae* isolate was identified by PCR amplification of the 16S rRNA gene using a Qiagen kit with the universal primers U341F and UA1406R. The PCR product was of the expected size (~1100 base pairs) on a 1.5 % agarose gel. The product was purified using QIA PCR purification kit and sent to GeneWiz (South Plainfield, N.J.) for sequencing. BLAST sequence alignment was performed at the NCBI web site (www.ncbi.nlm.nih.gov) with all top matched sequences pointing toward *E. undae*.

All growth experiments were carried out in duplicate (six tubes in total) and started by picking one colony of *S. aureus* or *E. undae* from a TSA plate and placing it in an Eppendorf tube that contained 1 ml of sterile TSB. Each Eppendorf tube was then vortexed for one minute. The six tubes were prepared as follows: tubes 1 and 2 contained 200 µl of the *S. aureus* suspension, tubes 3 and 4 contained 200 µl of the *E. undae* suspension and tubes 5 and 6 contained 100 µl of each suspension. Ciprofloxacin solution was added to one of each pair of tubes with tetracycline added to the other. TSB was added to bring the final volume of each tube to 10 ml

The initial growth experiments began with a low levels of ciprofloxacin (3.5 µg) or tetracycline (7.5 µg) in each tube. After reaching absorbance values at or above 1.0 at 600 nm a sterile loop was used to plate colonies from each tube on TSA plates. For all growth experiment average absorbance numbers were calculated for each pair of tubes and plotted as A600 nm versus

time. Once colonies were visible 12 new tubes were prepared as above but increasing the mass of ciprofloxacin to 4.25 µg per tube. The mass of tetracycline was held constant. This cycle was repeated a third time with 12 tubes increasing the mass of ciprofloxacin to the disc mass of 5 µg.

To test for the ability to evolve resistance to tetracycline, TSA plates from the last round of ciprofloxacin testing were now used to prepare 12, 10 ml tubes as above containing both bacterial isolates separate and in a mixed culture. The mass of ciprofloxacin was fixed at 5 µg per tube while the mass of tetracycline was increased to 10 µg. Two more rounds (15 µg and then 30 µg tetracycline) of the experiment were completed until the isolates had demonstrated their ability to grow at the mass of antibiotic indicated on the ciprofloxacin and tetracycline discs.

Results

S. aureus and *E. undae* were initially disc tested for susceptibility against ciprofloxacin (5 µg) and tetracycline (30 µg) as shown in Figure 1. The original *S. aureus* isolate had average zones of inhibition against ciprofloxacin of 25.5 mm and 26.5 mm against tetracycline indicating that it was susceptible to both antibiotics. The *E. undae* isolate had an average zone of inhibition of 26.0 mm with ciprofloxacin and 28.0 mm with tetracycline again indicating susceptibility.

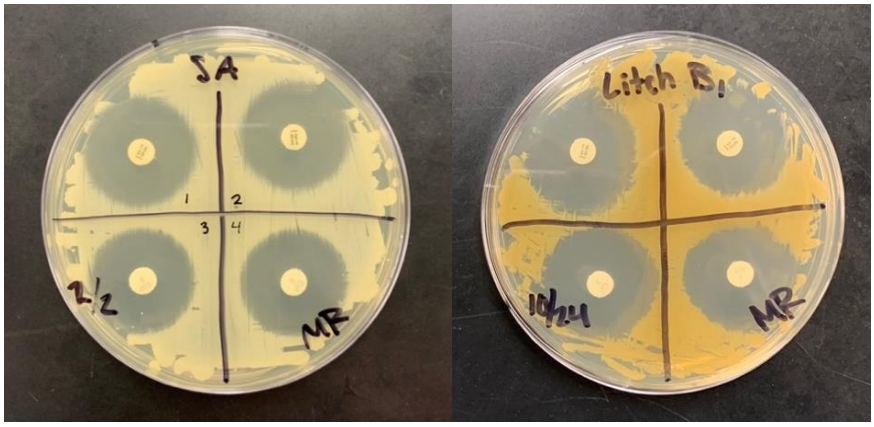

Figure 1. The left plate shows the original *S. aureus* against tetracycline disks in the top quadrants and ciprofloxacin disks in the bottom quadrants. The right plate shows the original *E. undae* against tetracycline disks in the top quadrants and ciprofloxacin disks in the bottom quadrants.

Figure 2 represents the growth of each isolate individually against the initial doses of each antibiotic as well as the mixed culture. *E. undae* was unable to grow against the minimal addition of tetracycline while the mixed cultures were two of the three (along with *S. aureus* with tetracycline) fastest growing tubes. A similar pattern was seen in Figure 3 as the mass of ciprofloxacin was increased – mixed cultures growing fastest, *E. undae* unable to grow in tetracycline but a decrease in the rate of growth of *S. aureus* in tetracycline was observed. At the disc value for ciprofloxacin (Figure 4) the growth pattern was similar. Figure 5 shows a similar disc diffusion assay as Figure 1 along with the decrease in the size of the zones of inhibition with ciprofloxacin discs. The zone of *S. aureus* after exposure to 5.0 µg of ciprofloxacin is 3.5 mm smaller than the average of the unexposed *S. aureus* found in

Figure 1. *E. undae's* zone of inhibition when tested against ciprofloxacin after exposure to 5.0µg of ciprofloxacin was 9 mm, 17 mm smaller than average unexposed *E. undae* that was tested in Figure 1. Colonies of both *S. aureus* and *E. undae* after exposure to 5.0 µg of ciprofloxacin were taken from TSA plates, vortexed in 1 mL of TSB and replated using a disposable loop on to a new TSA plate with four disks of ciprofloxacin. On average the mix of *S. aureus* and *E. undae* had a zone of inhibition of 13.5 mm against the ciprofloxacin disks.

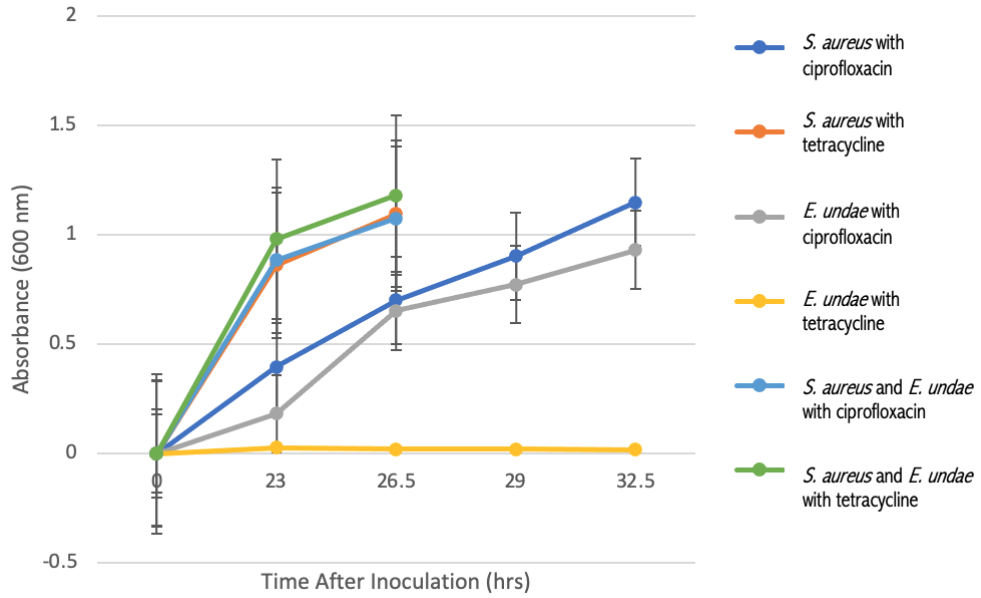

Figure 2. Graph of absorbance readings at 600 nm after inoculation of *S. aureus* and *E. undae* bacteria into 10 mL of TSB broth. Tubes contained 3.5 µg of ciprofloxacin or 7.5 µg of tetracycline. Standard error bars are shown.

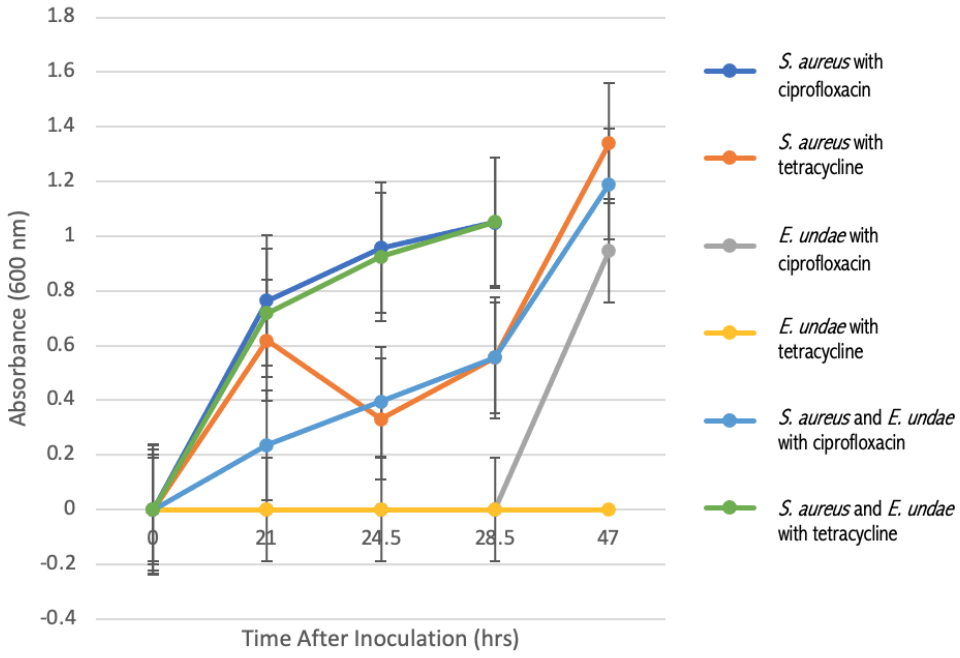

Figure 3. Graph of absorbance readings at 600 nm taken after inoculation of *S. aureus* and *E. undae* bacteria into 10 mL of TSB broth. Tubes contained 4.25 µg of ciprofloxacin or 7.5 µg of tetracycline. Standard error bars are shown.

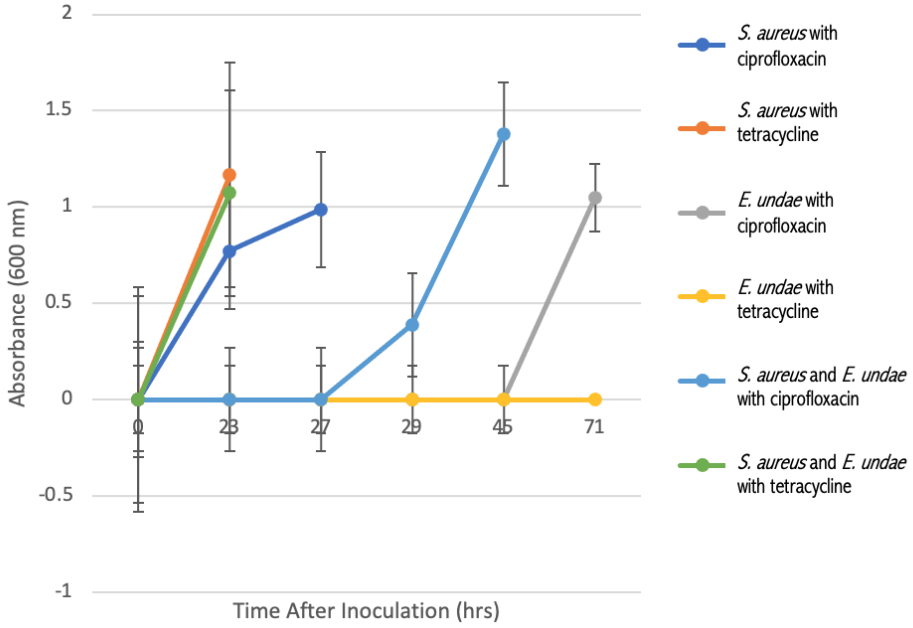

Figure 4. Graph of absorbance readings at 600 nm taken after inoculation of *S. aureus* and *E.undae* bacteria into 10 mL of TSB broth. Tubes contained 5.0 µg of Ciprofloxacin or 7.5 µg of tetracycline. Standard error bars are shown.

Figure 5. Top left plate shows original untested *S. aureus* zone of inhibition (29 mm) against a ciprofloxacin disk compared to *S. aureus* exposed to 5.0 µg of ciprofloxacin with a zone of inhibition of 22 mm. The bottom left plate shows original untested *E. undae* zone of inhibition (24 mm) against a ciprofloxacin disk and *E. undae* exposed to 5.0 µg of ciprofloxacin with a zone of inhibition of 9 mm. Top right and bottom right plates shows the mix of *S. aureus* and *E. undae* after exposure to 5.0 µg of ciprofloxacin tube against ciprofloxacin disks, the eight disks had an average zone of inhibition of 13.5 mm.

Results for the experiments increasing the amounts of tetracycline (ciprofloxacin level fixed) are shown in Figures 6 through 8. Here the *E. undae* isolate again demonstrated no ability to grow in the presence of tetracycline while the mixed culture (as well as the *S. aureus* isolate) got to absorbance levels at or above 1.0 within 24 hours. At higher levels of

ciprofloxacin, the *E. undae* isolate with ciprofloxacin did not begin to grow until 29-30 hours and at the highest levels of tetracycline the growth of the mixed culture was slower than that of the mixed culture versus ciprofloxacin.

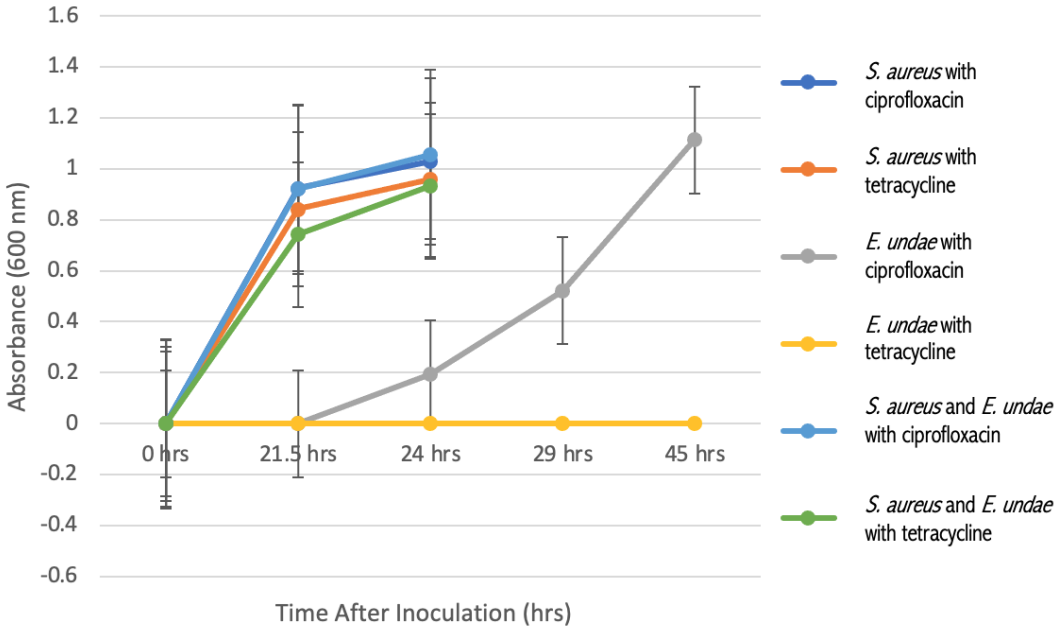

Figure 6. Graph of absorbance readings at 600 nm taken after inoculation of *S. aureus* and *E.undae* bacteria into 10 mL of TSB broth. Tubes contained 5.0µg of ciprofloxacin or 10µg of tetracycline. Standard error bars are shown.

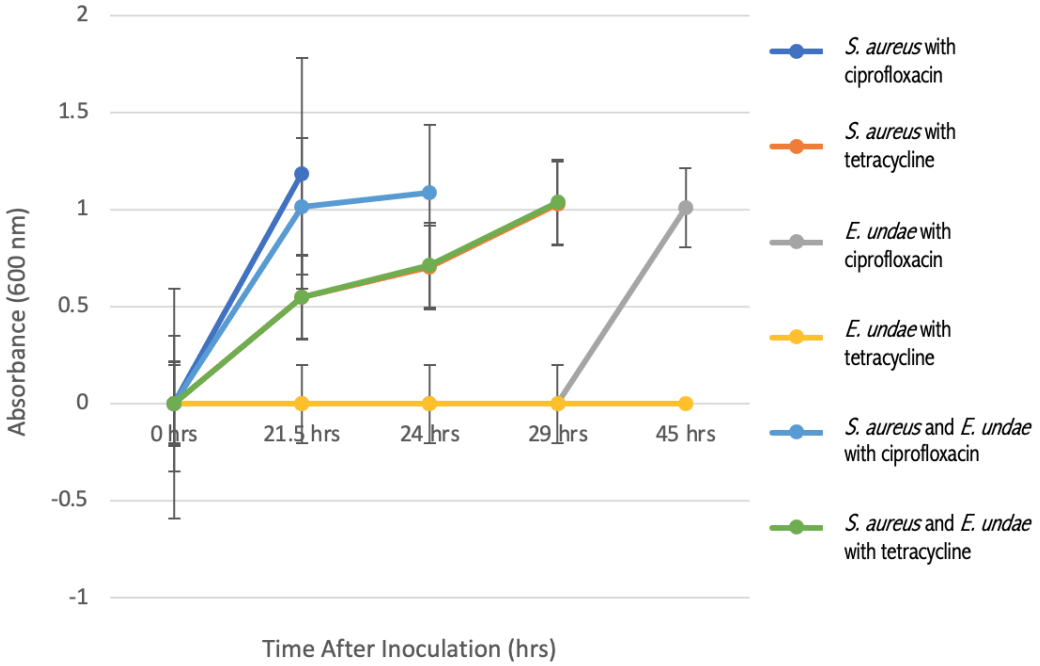

Figure7. Graph of absorbance readings at 600 nm taken after inoculation of *S.aureus* and *E.undae* bacteria into 10 mL of TSB broth. Tubes contained 5.0µg of ciprofloxacin or 15µg of tetracycline. Standard error bars are shown.

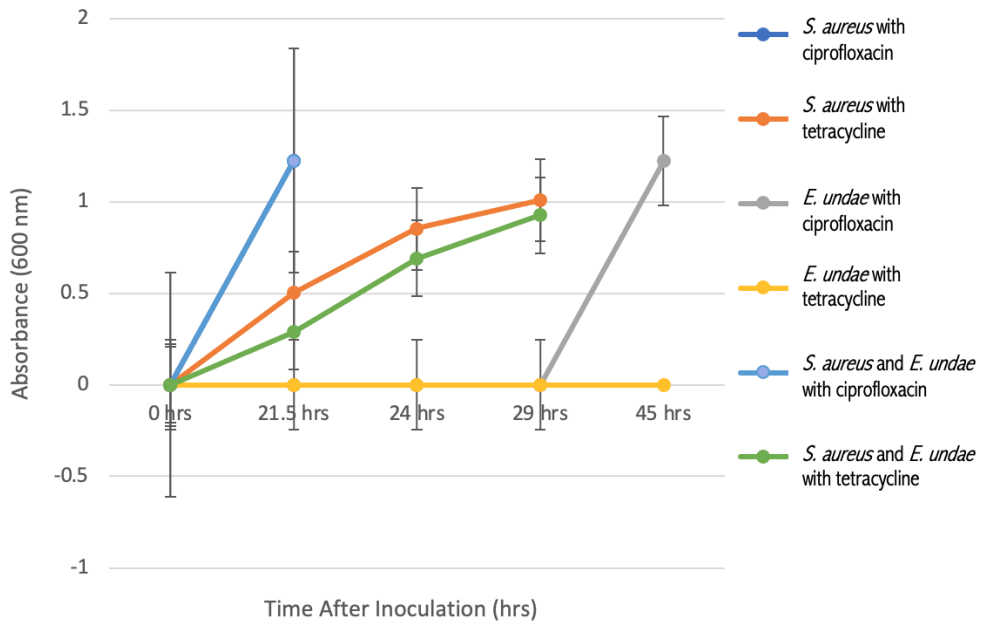

Figure 8. Graph of absorbance readings at 600 nm taken after inoculation of *S. aureus* and *E. undae* bacteria into 10 mL of TSB broth. Tubes contained 5.0 µg of ciprofloxacin or 30 µg of tetracycline. The line representing *S. aureus* with ciprofloxacin is hidden behind the mixed culture with ciprofloxacin because the numbers are too similar. Standard error bars are shown.

As a final experiment, isolates from the TSA plates after the exposure to the highest level of tetracycline were used to do a disc diffusion assay as shown in Figure 9. The results show the developed resistance to tetracycline for the *S. aureus* isolate and mixed cultures but also the inability of the *E. undae* isolate to develop resistance. In order to grow *E. undae* alone, a colony of *E. undae* was taken from the mixed bacteria exposed to tetracycline (30 µg) TSA plate and after a series of isolation plates was tested against a tetracycline disk and compared to the original sample used. *E. undae's* zone of inhibition when tested against tetracycline after exposure to 30µg of tetracycline was 42mm, 6 mm larger than unexposed *E. undae* that was tested simultaneously (Figure 9). The zone of *E. undae* after exposure to 30µg of tetracycline is 14mm larger than the average of the unexposed *E. undae* found in Figure 1 section.

After exposure to 30µg of tetracycline, *S. aureus* showed a new zone of inhibition of 8 mm. This zone is 33 mm smaller than what the *S. aureus* with no previous exposure to tetracycline showed when tested on the same plate at the same time (Figure 9). The zone of *S. aureus* after exposure to 30µg of tetracycline is 18.5 mm smaller than the average of the unexposed *S. aureus* found in Figure 1 section. The mixed culture of S. aureus and E. undae from the solution with 30µg of tetracycline was also exposed to the antibiotic disks. Colonies of *S. aureus* and *E. undae* were taken from the 30µg tetracycline TSA plates, vortexed in 1 mL of TSB and replated using a disposable loop on to a new TSA plate with four disks of tetracycline. On the plate of S. aureus and E. undae the average zone of inhibition was 11.5mm.

Figure 9. The left plate shows mixed bacteria taken from the 30 µg of tetracycline tube against tetracycline disks with an average zone of inhibition of 11.5 mm. The middle plate shows original untested *E. undae* with a zone of inhibition of 36 mm against a tetracycline disk compared to *E. undae* from the 30 µg of tetracycline tube with a zone of inhibition of 42 mm. The plate on the right shows original untested *S. aureus* with a zone of inhibition of 41 mm against a tetracycline disk compared to *S. aureus* from the 30 µg of tetracycline tube with a zone of inhibition of 8 mm.

Discussion

The purpose of this study was to investigate if two different bacteria can work together to develop resistance to ciprofloxacin and tetracycline. While *S. aureus* is known to produce biofilms and complement the growth of other microbes it was unknown if this could happen with an environmental isolate like *E. undae*. A study done on solutions for degrading polystyrene states that *Exiguobacterium* are involved in biofilm formation[11], the formation of a biofilm could be a potential mechanism for how *S. aureus* is complementing *E. undae*. Our results indicate that *S. aureus* could develop resistance to both ciprofloxacin and tetracycline but for *E. undae* to grow (or grow rapidly) it had to be in a mixed culture with *S. aureus*.

Ciprofloxacin is known as a treatment option for mixed infections or patients with predisposing conditions to Gram-negative infections[10]. Tetracycline is a broad-spectrum antibiotic, used to treat several acute infections[9]. The ability of both *S. aureus* and *E. undae* to decrease susceptibility to ciprofloxacin and for *S. aureus* to assist (complement) *E. undae* so that it could survive in a mixed culture are examples of the inherent dangers associated with antibiotic resistance in communal microbial settings. A study similar to our particular research created a synthetic community of two *Escherichia coli* strains where one was resistant to ampicillin and the other susceptible. They demonstrated the ability of a resistant strain to protect the susceptible strain through an enzyme called *β*-lactamase which degrades ampicillin[12]. Further studies on the interactions and methods of complementation between not only *S. aureus* and *E. undae* but additional organisms as well, could be a useful next step in understanding the means by which antibiotic resistance develops and spreads.

Beyond the general public, health professionals also need to be continually educated on the importance of not overprescribing, preventing the spread, and educating their patients on of resistant bacteria. With the increase in antibiotic resistant bacteria, we now have fewer effective antibiotics available than the pervious decade[13]. A study on patients in Turkey who had urinary tract infections (UTI) found 67.6% of the cultured bacteria resistant to ciprofloxacin[14]. Ciprofloxacin is one of the

most commonly prescribed medications for UTIs in Turkey and this could contribute to the high levels of antibiotic resistance. Our results align with what was observed in Turkey with regard to the development of resistance in bacteria exposed to ciprofloxacin.

There are multiple ways bacteria become resistant to antibiotics. Common mechanisms of resistance include changes to a target enzyme, enzyme inactivation of the drug, transformation of resistance genes, and active reflux pumps[15]. The misuse of and constant reexposure to antibiotics allows for the spread of these mechanisms. Research conducted on US adults found that of the 657 people who completed the survey 23 % obtained or knew someone else who obtained antibiotics without a prescription and less than half of the participants had ever talked with their doctor about antibiotic resistance[16]. Another similar study evaluated young adult's attitudes towards antibiotics and their knowledge of antimicrobial resistance. Researchers found that many young adults believe antibiotics to be a "fix all" solution. However, when provided with information about the dangers of antimicrobial resistance a correlation analyses showed a higher likelihood of appropriate antibiotic use[17]. These studies demonstrate how a small amount of knowledge could impact the publics use of antibiotics and potentially help combat the growing number of antibiotic resistant bacteria.

Our *E. undae* strain grew quite slowly as concentrations of ciprofloxacin increased but when combined with *S. aureus* grew at a much faster rate. *E. undae* had no ability to grow in the presence of tetracycline unless the laboratory *S. aureus* strain was present. This pattern was repeated when the amount of ciprofloxacin present was constant, but the amount of tetracycline was increased. Our data indicates that when the two strains were grown in a mixed culture, *S. aureus* contributed to the growth of *E. undae* in the presence of both ciprofloxacin and tetracycline. Strains of *S. aureus* have been reported to possess the genetic information necessary to produce tet(X), an enzyme that can inactivate tetracycline[18]. Several tetracycline inactivating enzymes have been reported in other pathogenic microbes. In one example, a recent study round 179 ciprofloxacin resistant strains of *Salmonella enterica* from human patients in Shanghai[19]. Most of these strains were resistant to additional antibiotics with 87 % of them being resistant to tetracycline. Another example comes from the gram-positive bacteria collected from the teaching hospital associated with Yasuj University of Medical Sciences that caused community acquired pneumonia, 43.1 % if the bacteria were found to be resistant to ciprofloxacin. *S. aureus* samples from that same study had a 61.5 % resistance pattern to ciprofloxacin, the highest percentage of all eight antibiotics the bacteria was tested against[20].

S. aureus, after repetitive exposure to increasing concentrations of both ciprofloxacin and tetracycline, decreased its susceptibility/increased its resistance to both antibiotics. In addition, demonstrated synergy or complementation was shown with the strain of *E. undae* in the presence of these same antibiotics. This demonstration of complementation or cooperativity is important in thinking about complementation, antibiotic resistance, and the mixed populations of microorganisms common in virtually every environmental setting.

Conclusion

The laboratory strain of *S. aureus* developed resistance to both ciprofloxacin and tetracycline. In addition, it could complement the growth of the *E. undae* isolate and allow growth as part of a mixed culture in tetracycline. Mixed cultures of both isolates generally grew faster than tubes where only one isolate or antibiotic was present.

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Conflict of Interest Statement:

The authors report no conflicts of interest related to this work.

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