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ISCI Capstone
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NSAIDs and Their Interactions with Bacterial Growth

Abstract:

Non-steroidal anti-inflammatory drugs (NSAIDs) are common medications that are frequently used to suppress symptoms of viral and bacterial infections. The increase in concern of antibiotic resistance has led researchers to study the effects of NSAIDs on bacterial growth or on common antibiotics. This study aims to determine whether commonly used NSAIDs, ibuprofen and acetaminophen, have any antibacterial properties that might help suppress bacterial growth. In conjunction, this study also looks to see whether the combination of NSAIDs and an antibiotic can further reduce bacterial growth than if an antibiotic is used alone. Our procedure consisted of lab-grown *Escherichia coli* (*E. coli*) that was mixed with ibuprofen (8ug/ml), acetaminophen (15ug/ml), or ampicillin at either 8 or 15ug/ml. These concentrations are based on typical blood serum levels of each respective drug. UV spectrophotometer was used to determine bacterial growth through absorbance values (600nm). The results show larger absorbance values (meaning greater bacterial growth) when ibuprofen and acetaminophen are present. Data also shows that when NSAIDs are used in combination with ampicillin, absorbance values would be greater than if ampicillin was used alone. Possible mechanisms leading to these results could include horizontal gene transfer in *E. coli* or inhibiting the antibiotic effects of ampicillin. Future research should focus on determining the primary mechanism NSAIDs use to promote bacterial growth or inhibit antibiotics.

Introduction:

The aim of this study is to determine whether common non-steroidal anti-inflammatory drugs (NSAIDs) contain any antibacterial properties that can complement the effects of antibiotics. With increasing prevalence of antibiotic resistance, understanding the relationship between NSAIDs and antibiotics is fundamental to the treatment of bacterial infections. Many types of NSAIDs are used to alleviate a wide range of symptoms, especially those present with bacterial infections. It is common that antibiotics and NSAIDs are used in combination with each other, making it more important to understand potential synergistic effects. Another relevant application is the increasing demand for drug repurposing. Manufacturing new drugs is a timely and costly process, so if already existing drugs can be considered pleiotropic (have one or more mechanisms), it would aid in treatment of diseases. According to Jelena Obad et al., suitability tests are required by pharmaceutical companies to ensure that drugs directly affect the targeted chemical, physical, and biological characteristic (2015). Many non-antibiotic drugs will initially show antimicrobial activity, but the pharmaceutical industry makes efforts to neutralize these attributes in order to maintain the intended purpose (Obad et al., 2015).

The relationship between antibiotics and NSAIDs has been extensively researched. However, there are still areas in this field of study that require further research. The mechanisms used by bacteria to acquire antibiotic resistant properties are poorly understood, especially when NSAIDs are used as an additional component to alleviate symptoms of bacterial infections. According to Alli Abdul Hussein and AL-Janabi, they found that ibuprofen and acetaminophen showed antibacterial activity in multiple strains of bacteria (2010). To determine the effects of

NSAIDs on bacterial growth, they used tubes of Mueller Hinton broth containing various concentrations of ibuprofen and acetaminophen. Their results displayed inhibited bacterial growth as the concentration of both NSAIDs increased. This study directly relates to understanding our experiment, because the authors analyzed the growth of bacteria in relation to NSAID exposure. On the contrary, other researchers found that NSAIDs may contribute to an increase in antibiotic resistance. According to Taru Verma et al., common NSAIDs may induce phenotypic antibiotic resistance in *Escherichia coli* (2018). The bacterial gene *marA* is a transcription factor that leads to the increased activity of efflux pumps that help remove antibiotics out of bacteria cells. The authors of this study determined that ibuprofen, acetaminophen, and acetylsalicylic acid (ASA) upregulates the *marA* gene in the bacteria *E. coli*.

Another study determined whether ibuprofen or the antibiotic pivmecillinam is more effective at treating *E. coli* infections. According to Ingvild Vik et al., the antibiotic pivmecillinam was more effective at treating the *E. coli* infection; however, ibuprofen still displayed positive effects towards the resolution of the infection (2018). They concluded that ibuprofen alone should not be a primary treatment for bacterial infections, but the combination of antibiotics and NSAIDs could provide beneficial results. Bacteria biofilm has also been a topic of interest when determining how some bacteria strains become resistant to antibiotics. Gram-negative bacteria, like *E. coli*, produce a biofilm coating that wraps around the bacteria cell wall and acts as a protective mechanism to ensure bacteria survivability. A study conducted by Cláudia Leão et al., suggested that NSAIDs inhibit biofilm formation through disrupted DNA synthesis that codes for replication and membrane repair of the biofilm (2020). This disruption would allow more antimicrobial agents to enter the bacteria cells, promoting greater effectiveness of antibiotics.

The present study investigates whether ibuprofen or acetaminophen have any antibacterial properties. Additionally, this study would like to see whether the combination of ibuprofen or acetaminophen with ampicillin will lead to greater reduction in bacteria growth. To measure bacterial growth, absorbance values will be recorded using a spectrophotometer at 600nm. It is hypothesized that absorbance values will lower when NSAIDs are present with *E. coli*. Additionally, it is predicted that absorbance values will be even lower when the NSAIDs are combined with the antibiotic ampicillin.

Methods/Materials:

The bacteria used in this experiment was *Escherichia coli* (*E. Coli*). The *E. coli* was provided by the microbiology lab at Saint John's University. *E. Coli* is a gram-negative bacteria found in the intestines of humans and animals. The *E. coli* strain obtained for this experiment was lab grown, having the benefit of limited antibiotic resistant properties. The NSAIDs used in this experiment were ibuprofen and acetaminophen. Stock solutions of these NSAIDs were prepared by crushing an over-the-counter 200mg oral tablet of ibuprofen and a 500mg oral tablet of Tylenol (acetaminophen) using a mortar and pestle. Once crushed to powder, it was dissolved in equal parts of 95% ethanol and 1N hydrochloric acid (HCl), 50mL each. HCl was used to resemble the gastrointestinal environment that the NSAID tablets would be exposed to if ingested. Ethanol was used as a solvent due to its ability to dissolve NSAID tablets. A similar study conducted by James Riordan et al., used ethanol to dissolve their NSAIDs which led us to use the same technique (2011). These solutions were mixed and heated in a microwave until the powder was completely dissolved. 10mL of each NSAID solution was extracted and then pushed through a sterile filter to remove any impurities that may have been present.

Typical blood concentration levels of ibuprofen are between 6-10 μ g/mL and acetaminophen between 10-20 μ g/mL. The concentration equation ($Concentration_1 \times Volume_1 = Concentration_2 \times Volume_2$) was used to determine the appropriate number of microliters needed to achieve a concentration 8 μ g/mL of ibuprofen and 15 μ g/mL of acetaminophen. The total volume within the test tube would be 3mL. Based on the concentration equation, it was determined that 12 μ L of ibuprofen and 9 μ L of acetaminophen should be used through the experiment. The antibiotic used in this experiment was ampicillin. Stock solution of ampicillin was obtained at a concentration of 10mg/mL. Again, the concentration equation was used to calculate the amount needed to reach typical blood concentrations of both 8 and 15 μ g/mL. The second experiment used 2.4 μ L of ampicillin to achieve a concentration of 8 μ g/mL, and the third experiment used 4.5 μ L of ampicillin to achieve a concentration of 15 μ g/mL.

The first experiment only required E. coli, Trypticase soy broth (TSB), and NSAID stock solutions. The decision to use TSB broth was based on a study conducted by Ileana-Andreea Ratiu et al (2017). Researchers compared multiple broth solutions and determined that TSB is a complex broth medium that effectively grows pathogenic bacteria, like E. coli (Ratiu et al., 2017). 3mL of TSB and 50 μ L of E. coli were added to 15 test tubes and then separated into 3 sets of 5. The first set only contained E. coli and TSB. The second set received an additional 12 μ L of ibuprofen stock solution. And the third set received 9 μ L of acetaminophen stock solution. All test tubes were placed in an incubator for 24 hours at 37°C. We chose these settings based on an experiment designed by Noton Dutta et al., that found these parameters were optimal for bacteria growth (2007).

The second experiment required E. coli, TSB, NSAID stock solutions, and ampicillin (8 μ g/mL). 48 test tubes were separated into 3 sets of 16, with the first set containing 3mL of TSB, 50 μ L of E. coli, and 2.4 μ L of ampicillin. The second set received an additional 12 μ L of ibuprofen stock solution and the third set received an additional 9 μ L of acetaminophen stock solution. All 48 test tubes were placed in an incubator for 24 hours at 37°C.

The third experiment required E. coli, TSB, NSAID stock solutions, and ampicillin (15 μ g/mL). 45 test tubes were separated into 3 sets of 15, with the first set containing 3mL of TSB, 50 μ L of E. coli, and 4.5 μ L of ampicillin. The second set received an additional 12 μ L of ibuprofen stock solution and the third set received an additional 9 μ L of acetaminophen stock solution. All 45 test tubes were placed in an incubator for 24 hours at 37°C.

After the incubation period, the test tubes were removed from the incubator and analyzed using a UV-Vis spectrophotometer set at 600nm. Individually, 1mL of each test tube was extracted and placed in a 1cm cuvette. Absorbance readings were obtained and compiled in a Microsoft Excel spreadsheet. The average absorbance and standard deviation values were calculated within Excel for each set within each experiment. The standard deviation of the mean was calculated using the standard deviation divided by the square root of how many tubes were in each set. Two t-tests were calculated, one for experiment 2 and the other for experiment 3, to determine p-values. The t-test for experiment 2 compared ibuprofen with ampicillin (8 μ g/mL) to ampicillin alone. The t-test for experiment 3 compared acetaminophen with ampicillin (15 μ g/mL) to ampicillin alone.

Results/Analysis:

Experiment 1: NSAIDs and E. Coli

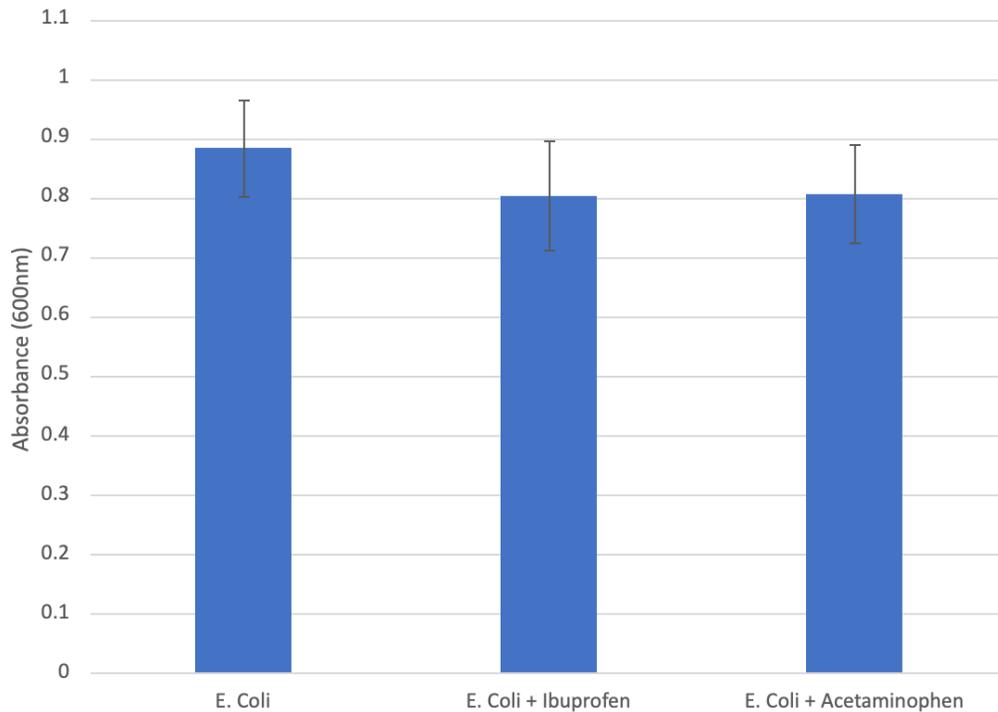


Figure 1. Displays the average absorbance of the bacteria (*Escherichia Coli*) when acetaminophen (15µg/mL) or ibuprofen (8µg/mL) are present. Absorbance is directly proportional to concentration, indicating higher absorbance corresponds to more bacterial growth. Error bars represent standard deviation of mean, n=5 per column.

The first experiment determined if ibuprofen or acetaminophen had any antibacterial properties if used independently. The average absorbance of *E. coli* without any treatment was 0.885 ± 0.013 . When ibuprofen was added to *E. coli* the average absorbance was 0.804 ± 0.091 . Comparatively, acetaminophen had similar results to ibuprofen with an average absorbance of 0.807 ± 0.082 . No t-test was used to establish any statistical significance due to the large overlap of the error bars, possibly due to a small sample size. Based on figure 1, there is no evidence that shows commonly used NSAIDs had any antibacterial properties at concentrations of 15µg/mL of acetaminophen or 8µg/mL of ibuprofen.

Experiment 2: NSAIDs, *E. Coli*, Ampicillin (8µg/mL)

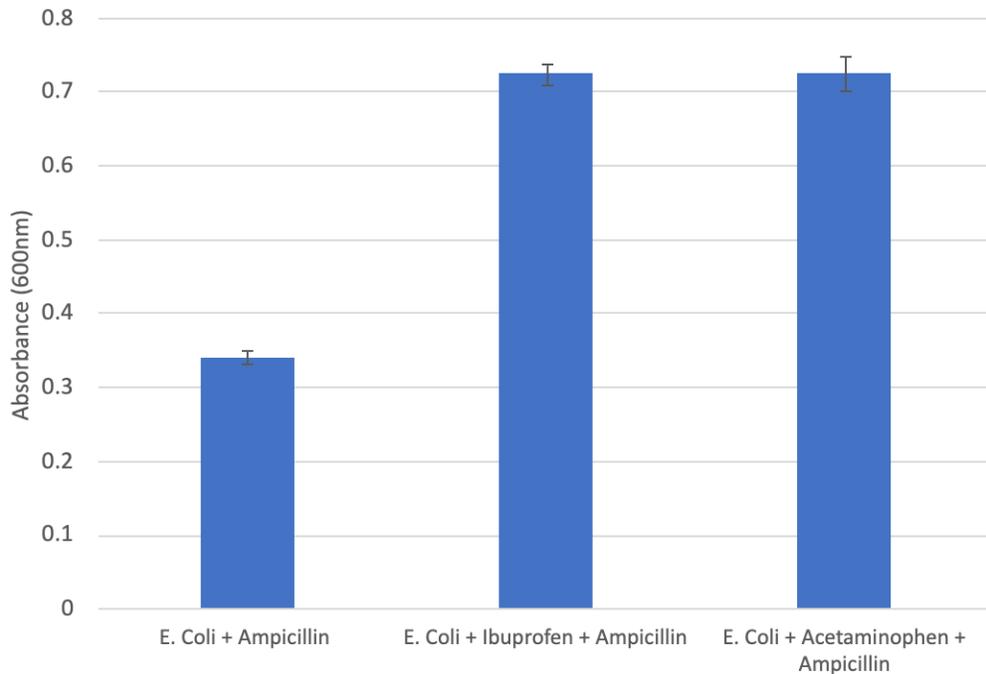


Figure 2. Compares the average absorbance of the bacteria (*Escherichia Coli*) when acetaminophen (15µg/mL) or ibuprofen (8µg/mL) is combined with ampicillin (8µg/mL). Absorbance is directly proportional to concentration, indicating higher absorbance corresponds to more bacterial growth. Error bars represent standard deviation of mean, n=16 per column.

The second experiment determined whether the combination of NSAIDs and ampicillin, at 8µg/mL, had any effect on bacterial growth. When ampicillin was used independently with *E. coli* the average absorbance was 0.340 ± 0.009 . However, when ibuprofen or acetaminophen is used in conjunction with ampicillin the average absorbance values were significantly higher. The average absorbance of ibuprofen with ampicillin was 0.723 ± 0.015 . Similarly, when acetaminophen was used with ampicillin, an average absorbance value of 0.724 ± 0.023 was produced. Interestingly, both treatments produced similar absorbance values and had comparable deviations. A two-sample t-test was calculated to determine the significance between ampicillin used independently and ampicillin used with ibuprofen. The p-value was $2.91e-17$, indicating statistical significance and disproving the null hypothesis.

Experiment 3: NSAIDs, *E. Coli*, Ampicillin (15µg/mL)

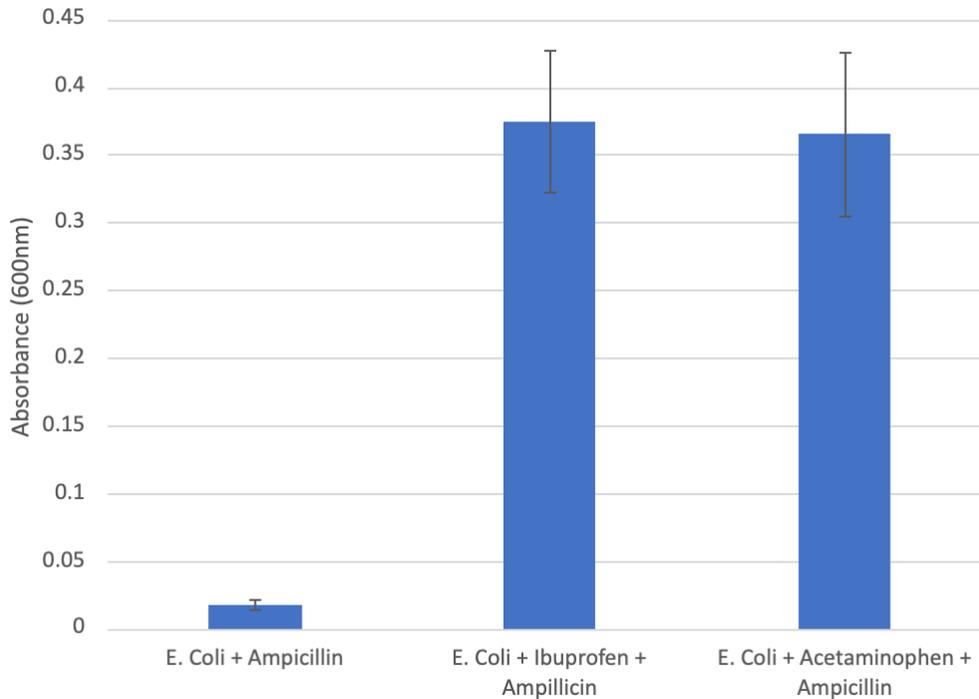


Figure 3. Compares the average absorbance of the bacteria (*Escherichia Coli*) when acetaminophen (15µg/mL) or ibuprofen (8µg/mL) is combined with ampicillin (15µg/mL). Absorbance is directly proportional to concentration, indicating higher absorbance corresponds to more bacterial growth. Error bars represent standard deviation of mean, n=15 per column.

The third experiment was identical to the second experiment except for the concentration of ampicillin (15µg/mL). A higher concentration was used because individual blood serum concentration of ampicillin can have large variations. When ampicillin was used independently with *E. coli* the average absorbance was 0.018 ± 0.004 . However, when ibuprofen or acetaminophen was used in conjunction with ampicillin the average absorbance values were significantly higher, similarly seen in Figure 2. The average absorbance of ibuprofen with ampicillin was 0.375 ± 0.052 . Similarly, when acetaminophen was used with ampicillin, an average absorbance value of 0.365 ± 0.061 was produced. Interestingly, both treatments produced similar absorbance value and had comparable deviations. A two-sample t-test was calculated to determine the significance between ampicillin used independently and ampicillin used with acetaminophen. The p-value was $7.68e-5$, indicating statistical significance and disproving the null hypothesis.

There are multiple potential causes for the results seen in our three experiments. In a study conducted by Agnieszka Laudy et al., they determined the activity of MDR efflux pumps integrated within bacteria membranes (2016). The function of these pumps is to remove toxic substances, like antibiotics, and increase bacterial survivability. Researchers also determined that ibuprofen and acetaminophen could potentially be substrates for the efflux pump, giving them limited or no antibacterial properties (Laudy, 2016). These findings can explain why there is no significant difference in absorbance values when NSAIDs are used independently (Figure 1).

Another possible explanation for our results is the concept of horizontal gene transfer that helps promote antibiotic resistance. In an experiment conducted by Yuqian Jia et al., they found that acetaminophen specifically can increase the rate and efficiency of horizontal gene transfer (2021). Horizontal gene transfer is a mechanism that bacteria cells use to help share DNA during their reproduction phase (Jia et al., 2021). One common antibiotic resistant gene, MarA, is known to participate in horizontal gene transfer making bacteria cells less susceptible to

ampicillin (Jia et al., 2021). Our results show higher absorbance values when NSAIDs are used in combination with ampicillin at concentrations of 8µg/mL and 15µg/mL (Figure 2,3). These results can indicate the presence of horizontal gene transfer throughout the 24hr incubation period.

Conclusion:

The combination of NSAIDs and antibiotics are frequently used to help treat bacterial infections. It is vital that researchers and clinicians understand any interaction that may exist between two of the most utilized drugs. It is important to understand the limitations present within this study. We were not able to gene sequence bacterial strains, so no conclusion can be drawn on the definite mechanism NSAIDs use to increase the survivability of bacteria cells. It should also be noted that other research studies incubated their bacterial cultures longer than 24hr. Future research should include extended incubation periods as well as gene sequencing. To rule out any possible chemical alterations between the ethanol/HCL mixture and ampicillin, additional trials can be conducted. Future steps should include a fourth set of test tubes that contain broth, E. coli, ethanol/HCl mixture, and ampicillin.

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