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Article

The effect of bioenhancers on ampicillin-sulbactam as a treatment against *A. baumannii*

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SUMMARY

As more bacteria develop antibiotic resistance, bioenhancers – substances that enhance antibiotic performance – are emerging as potential solutions. However, the effectiveness of bioenhancers has not been measured on highly antibiotic-resistant pathogens. We hypothesized that the bioenhancer piperine, derived from black pepper, combined with the drug ampicillin-sulbactam, would lead to decreased growth of *Acinetobacter baumannii*, a highly resistant bacterium, compared to the drug alone. Broth dilution was used to find the minimum concentration of drug needed to inhibit three strains of *A. baumannii*, with *Escherichia coli* as a control, providing a baseline for the strain's resistance levels. The strains were exposed to four treatments: varying concentrations of ampicillin-sulbactam alone, piperine alone, a combination of ampicillin-sulbactam and piperine, and no treatment to analyze the antibiotic-bioenhancer synergy. Treatments were also incubated with macrophages, cells of the mammalian innate immune response, measure drug efficacy under conditions mimicking early stages of infection. The results indicated that combining piperine with ampicillin-sulbactam at 64 mg/L and 32 mg/L reduced bacterial growth for two *A. baumannii* strains compared to treatment with antibiotic alone. However, the third strain had no significant difference in bacterial growth. However, at a drug concentration of 16 mg/L, the addition of piperine led to significantly reduced bacterial growth for all three strains ($p < 0.001$, $p < 0.0001$, $p < 0.0001$). These studies showed that piperine enhanced ampicillin-sulbactam efficacy against *A. baumannii*. The addition of piperine led to lower minimum inhibitory concentrations of the antibiotic against drug-resistant *A. baumannii*.

INTRODUCTION

Infections caused by carbapenem-resistant *Acinetobacter baumannii* hospitalize thousands and cost hundreds of millions of dollars in healthcare costs yearly in the United States (1). This gram-negative bacterium is particularly dangerous due to its high levels of resistance to many antibiotics, including fluoroquinolones and β-lactams (1). Infections caused by *Acinetobacter* species are especially prevalent in healthcare settings, leading to the inclusion of this bacterium in the CDC's list of urgent antibiotic resistance threats (1). This is due to their ability to develop multiple resistance mechanisms to survive higher concentrations of antimicrobials used to treat these infections, resulting in increased treatment failure rates (2). The combination of ampicillin and sulbactam is one of the best antimicrobials for severe *Acinetobacter* infections (3). Ampicillin is a β-lactam that works by interfering with bacterial cell wall synthesis. However, ampicillin alone is inactivated by β-lactamases in *A. baumannii* due to the pathogen's strong antibiotic resistance (3). Sulbactam is a β-lactamase inhibitor that prevents the bacteria from destroying the ampicillin, theoretically solving this issue (3). Nevertheless, *A. baumannii* has developed intermediate levels of resistance to ampicillin-sulbactam (1). Only in very high doses does the drug have the potential to inhibit the growth of *A. baumannii* (4). High doses make the treatment of infections more costly and less accessible, and higher doses are also correlated with increased side effects, lowering patient compliance (5). Therefore, improved therapies are urgently needed to treat A. baumannii infections adequately.

An emerging solution to antibiotic resistance in bacteria is bioenhancers. These compounds found in nature can be combined with antibiotics to improve their ability to treat bacterial infections. Bioenhancers have the potential to drastically reduce the amount of drugs needed to treat infections (6). Bioenhancers have historically been studied based on their ability to increase antibiotic bioavailability, or the amount of drug that successfully enters the body's circulation, thereby decreasing resistance (7). One of the most common bioenhancers is piperine, found in the *Piper nigrum* extract. Piperine inhibits human P-glycoprotein, a protein efflux pump expressed in multiple tissues throughout the body that actively transports small molecules, including antibiotics and other drugs, out of the cell (8). Inhibition of P-glycoprotein by piperine has increased drug bioavailability (9). However, the ability of a bioenhancer like piperine to directly inhibit bacterial growth has not been explored. Many bacteria possess efflux pumps that are key components of their resistance mechanisms.

Along with β-lactamases, *A. baumannii* possesses the AdeB efflux pump belonging to the Resistance-Nodulation- Division (RND) efflux pump family that binds intracellular antibiotics and transports them out of the cell, providing an intrinsic resistance to β-lactam drugs such as penicillin (2,10). This directly contributes to growing antibiotic resistance within the pathogen. It is known that piperine can inhibit efflux pumps in less resistant bacteria, such as the NorA efflux transporter present in *Staphylococcus aureus,* a gram-positive bacterium (11,12). However, efflux pumps of *A. baumannii* and other gram-negative bacteria

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employ protein channels extending from the inner membrane across the periplasm to the outer membrane envelopes (10). The RND efflux pump significantly contributes to resistance in gram-negative bacteria, including *A. baumannii* (13). This allows gram-negative bacteria to resist a variety of antibiotics in comparison. These antibacterial mechanisms within piperine are largely unexplored for more antibiotic-resistant bacteria such as *A. baumannii.* It is unknown if the antimicrobial effects of bioenhancers will be effective against Multidrug-resistant organisms like *A. baumannii.* We hypothesized that piperine may increase the antimicrobial activity of the β lactam drug ampicillin in resistant *A. baumannii* expressing the AdeB efflux pump (2). This would allow more drugs to affect the bacteria and theoretically reduce the antibiotic dosages.

In this work, we aimed to combine piperine with ampicillin-sulbactam to measure the effectiveness of the combined treatment in inhibiting the growth of *A. baumannii* in bacterial culture and within infected cells. We hypothesized that adding piperine to ampicillin-sulbactam would lead to less bacterial growth when compared to the drug alone. Our results showed that when *A. baumannii* was treated with the drug, the addition of piperine led to a significant reduction in bacterial growth for a range of *A. baumannii* strains with varied resistance levels. However, preliminary studies in mammalian cells investigating piperine's impact on bacterial growth were inconclusive. Follow-up studies with additional replicates are needed in this regard.

RESULTS

Effect of Piperine and Ampicillin-Sulbactam in Bacterial Culture

To study the effect of piperine on the antibacterial activity of ampicillin-sulbactam, we utilized a total of four bacterial strains in the experiments: three strains of multidrug-resistant *A. baumannii,* labeled as strains A, B, and C, as well as one strain of laboratory stock *E. coli*, labeled strain E, as a control. Multiple strains were used because of their varying levels of antibiotic resistance. This allowed us to understand the impact of our treatment groups across a spectrum of resistance in *A. baumannii.* We used a broth dilution assay to obtain a qualitative idea of how drug-resistant the strains were in comparison to one another. For this assay, each strain was exposed in an identical manner to increasing concentrations of ampicillin-sulbactam, to quantify the amount of drug needed to inhibit 50% of bacteria measured by optical density. We found that among the strains of *A. baumannii,* strain A was the most resistant, strain B was moderately resistant, and strain C was the least resistant. Strain E, the control, was much less resistant than the three *A. baumannii* strains. We then exposed these bacterial strains to four different treatments in a microplate. These included broth media alone as negative control without any treatment, a positive control with ampicillin-sulbactam alone, a group exposed to piperine alone, and a group exposed to a combination of ampicillin-sulbactam and piperine. Within these four conditions, the bacteria were exposed to varying concentrations 64 mg/L down to 16 mg/L of ampicillin-sulbactam alone or with 4 mg/L of piperine. This portion of the study allowed us to compare the efficacy of the antibiotic alone with that of the combination treatment.

Bacterial growth was measured using optical density (OD 600) via spectrophotometry 24 hours after initiating culture. Against strain A, the most resistant *A. baumannii* strain, the addition of piperine to ampicillin-sulbactam did not change bacterial growth when compared to ampicillin-sulbactam alone at a drug concentration of 64 mg/L (one-way ANOVA test with Tukey HSD, $p > 0.05$) and 32 mg/L (one-way ANOVA test with Tukey HSD, $p > 0.05$) but significantly decreased bacterial growth when the drug concentration was 16 mg/L (one-way ANOVA test with Tukey HSD, $p = 0.0003$, **Figure 1).** Against strain B, which

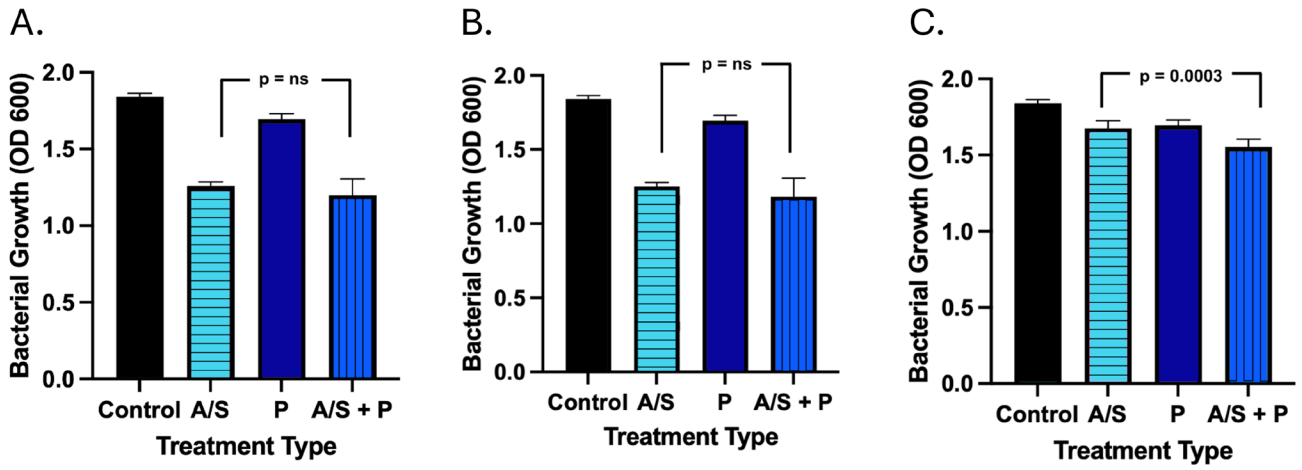

Figure 1. Piperine and ampicillin-sulbactam slightly improved inhibition of *A. baumannii* strain A (H72721) compared to the drug alone. Bar graphs comparing bacterial growth for the four treatment groups at 64 mg/L (Panel A), 32 mg/L (Panel B) and 16 mg/L (Panel C) of ampicillin-sulbactam. Strain A was exposed to just antibiotic (A/S), piperine alone (P), or the combination treatment with varying drug concentrations (A/S + P) (for all graphs, n = 16 (control), n = 3 (A/S, A/S + P), n = 24 (P)). Optical density was measured after 24h. One-way ANOVA with Tukey HSD statistical analysis is shown between antibiotic alone and the combination treatment

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was moderately resistant, the addition of piperine to ampicillin-sulbactam led to a statistically significant decrease in bacterial growth at drug concentrations of 64 mg/L (one-way ANOVA test with Tukey HSD, $p < 0.0001$), 32 mg/L (one-way ANOVA test with Tukey HSD, $p = 0.0171$), and 16 mg/L (one-way ANOVA test with Tukey HSD, $p < 0.0001$) when compared to the performance of the antibiotic alone **(Figure 2).** Against strain C, the least resistant *A. baumannii* strain, the addition of piperine to the ampicillin-sulbactam significantly inhibited bacterial growth compared to the drug alone at a drug concentration of 64 mg/L (one-way ANOVA test with Tukey HSD, $p = 0.0005$), 32 mg/L (one- way ANOVA test with Tukey HSD, $p = 0.0002$), and 16 mg/L ($p < 0.0001$, **Figure 3).** Lastly, against the least resistant *E. coli* strain, which required much lower concentrations of ampicillin-sulbactam to inhibit bacterial growth, the addition of piperine to the drug did not result in a significant decrease in bacterial growth compared to ampicillin-sulbactam alone at a drug concentration of 8 mg/L (one-way ANOVA test with Tukey HSD, $p > 0.05$). However, at drug concentrations of 4 mg/L and 2 mg/L, this decrease in bacterial growth was significant (one-way ANOVA test with Tukey HSD, $p < 0.0001$, $p < 0.0001$, **Figure 4).**

Effect of Piperine with Ampicillin-Sulbactam treatment of infected macrophages

In these studies, live RAW 264.7 murine macrophage cell-line cultures were infected with either *A. baumannii* strain A or strain B. The same four treatment groups (no treatment, ampicillin-sulbactam alone, piperine alone, and ampicillin-sulbactam combined with piperine) were used to treat the cells. To maintain consistency with the amount of drug tested in broth culture, infected macrophages were treated with ampicillin-sulbactam concentrations of 64 mg/L for strain A and 32 mg/L for strain B infected cultures. We recorded the number of bacteria present within macrophages using the multiplicity of infection (MOI) or the ratio of bacteria visualized within macrophages after 12 hours of treatment. For Raw cells infected with *A. baumannii* strains A and B, the difference in MOI between ampicillin sulbactam and piperine and ampicillin-sulbactam alone was not significant (one-way ANOVA test with Tukey HSD, $p > 0.05$, $p > 0.05$, **Figure 5).**

DISCUSSION

In the bacterial culture studies, we observed general trends across the strains of varying resistance. Though the addition of piperine had a negligible impact on bacterial growth of all three *Acinetobacter* strains investigated at high antibiotic concentrations, the piperine-induced increase in antimicrobial activity was greater at the lowest drug concentration tested for all three *A. baumannii* strains. The *E. coli* strain also exhibited this trend, as the piperine and drug combination inhibited bacterial growth most effectively at the lowest antibiotic concentration studied (2 mg/L). This indicates that when piperine is added to the antibiotic, it acts synergistically at lower antibiotic concentrations and has the potential to lower the amount of drug administered for treatment. Overall, adding piperine to ampicillin-sulbactam resulted in less bacterial growth than the drug alone. This supports our initial hypothesis that piperine acts as a bioenhancer of antimicrobials. A direct bacterial enumeration could have been used to confirm bacterial counts, such as serial dilution of culture lysates and direct plating of aliquots onto agar media to enumerate viable colony forming units in each treatment group.

A limitation of our experiment with mammalian cells was the number of replicates we were able to study. Our data was severely limited due to contamination of the cell media. This significantly reduced our resource availability, resulting in fewer replicates than we would have preferred. The results of

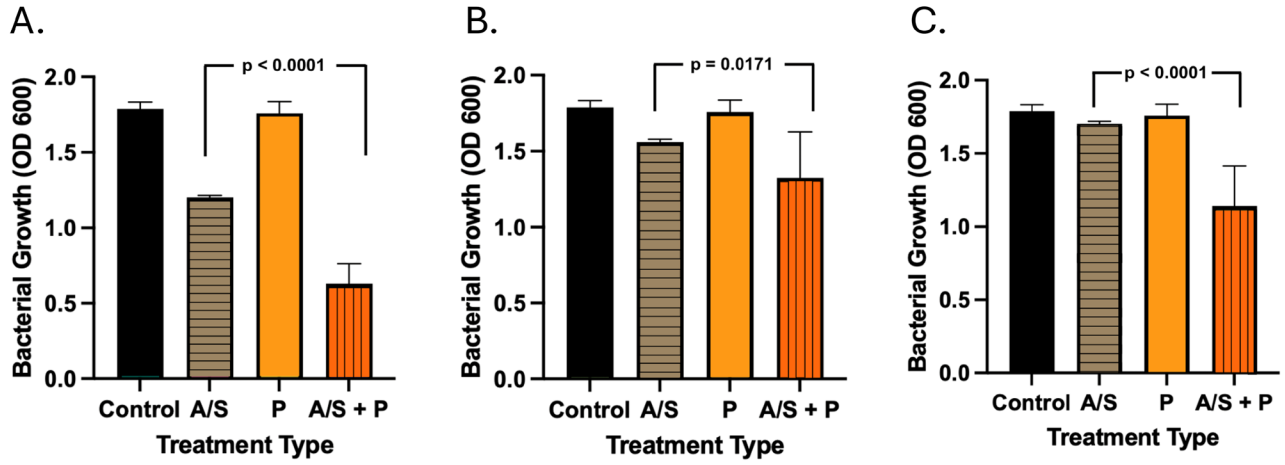

Figure 2. Piperine and ampicillin-sulbactam significantly inhibited *A. baumannii* strain B (BC-5) compared to ampicillin-sulbactam alone. Bar graphs comparing bacterial growth for the four treatment groups at 64 mg/L (Panel A), 32 mg/L (Panel B) and 16 mg/L (Panel C) of ampicillin-sulbactam (for all graphs, n = 16 (control), n = 3 (A/S, A/S + P), n = 24 (P)). Strain B was exposed to just antibiotics, piperine, and a combination treatment with varying drug concentrations. Optical density was measured after 24h. One-way ANOVA with Tukey HSD statistical analysis is shown between antibiotics alone and the combination treatment.

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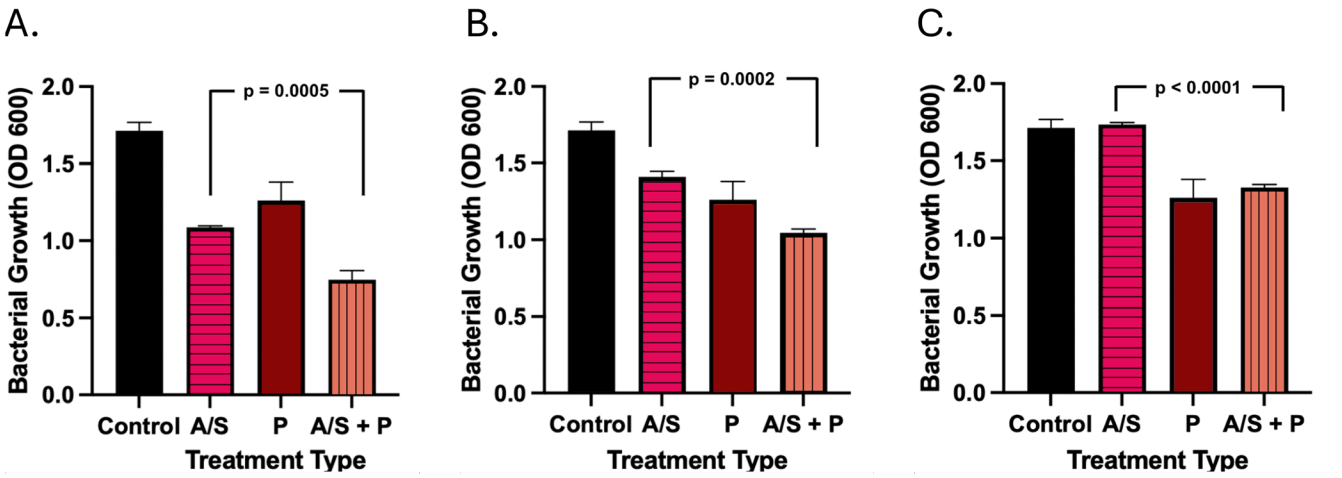

Figure 3. Piperine and ampicillin-sulbactam significantly inhibited *A. baumannii* strain C (AB5075-UW) compared to ampicillin-sulbactam alone. Bar graphs comparing bacterial growth for the four treatment groups at 64 mg/L (Panel A), 32 mg/L (Panel B) and 16 mg/L (Panel C) ampicillin-sulbactam concentrations (for all graphs, n = 16 (control), n = 3 (A/S, A/S + P), n = 24 (P)). Strain C was exposed to antibiotics, piperine, and a combination treatment with varying drug concentrations. Optical density was measured after 24h. One-way ANOVA with Tukey HSD statistical analysis is shown between antibiotics alone and the combination treatment.

this experiment were quite variable due to the limited data we were able to collect. Because a visible drop in bacterial growth was observed, more data would need to be collected to reach a conclusive result on the impact of piperine within mammalian cells. The method used to determine the MOI could also be an experimental limitation. During this procedure, visualizing and counting individual bacteria within macrophages using brightfield microscopy is sensitive to human judgment, so it was prone to errors that may have influenced results.

Additionally, researchers were not blinded to the identity of the samples, leaving room for unconscious biases during the quantification process. This last study remains inconclusive and requires further testing and a more accurate data collection method. Despite these limitations, the results of our overall study indicate that piperine could be a crucial component to combat antibiotic resistance. In bacterial culture, it improved the efficacy of ampicillin-sulbactam, helping it better combat *A. baumannii* and reducing the amount of antibiotic needed to have the same effect. Piperine can potentially improve treatment of antibiotic-resistant infectious diseases by facilitating higher antimicrobial activity and lowering the amount of antibiotics needed.

In the future, along with performing additional experimentation in vitro in mammalian cells, we would like to perform in-vivo experiments to see if piperine's addition remains viable in an animal model. Additionally, we want to experiment with other bioenhancers, such as ginger or curcumin. Other bioenhancers may have different mechanisms by which they combat resistance that may be more or less effective against highly resistant pathogens like *A. baumannii.* Discovery into which bioenhancers are efficacious against *A. baumannii* would also be highly valuable. Finally, experiments should be done to confirm the mechanism by which piperine enhances the effects of ampicillin-sulbactam. The results suggest that the piperine may act by inhibiting the efflux pump of *A. baumannii*, but further experimentation would have to be done to test the mechanisms involved. Piperine's ability to combat bacterial growth of resistant *A. baumannii* strains was previously unknown, and our results show that when combined with ampicillin-sulbactam, it could significantly prevent bacterial growth and allow for lower drug doses for treatment. Additionally, piperine is a widely studied therapeutic with additional pleiotropic effects, reaffirming its viability as a bioenhancer (14).

While more studies are needed to explore the viability of this treatment in vitro and in vivo, this study demonstrates the potential of piperine to work synergistically with an antibiotic to kill more bacteria and reduce the required concentration of antibiotic. These aspects make piperine a promising compound for dealing with growing antibiotic resistance in various pathogens. The outcome of this research could provide a less costly and more effective treatment to combat *A. baumannii* infections, which are becoming an urgent threat worldwide.

MATERIALS AND METHODS

Minimum Inhibitory Concentration Measurement

Three commercially available strains (American Type Culture Collection) of *A. baumannii* (H72721, BC-5, AB5075-UW) as well as laboratory stock *E. coli* strain JM109 grown in Brain Heart Infusion (BHI) broth (Fisher Scientific). These bacterial suspensions were adjusted to an optical density (OD_{600}) of 0.08 – 0.10, representing about 1 x 10^8 colony-forming units (CFU)/mL. A broth dilution assay determined the minimum inhibitory concentration (MIC), the antibiotic concentration inhibiting 50% of bacterial growth for each strain. Optical density values from wells with no drug represented 100% or maximal growth to calculate 50% inhibition values. The stock solution of ampicillin-sulbactam (Sigma-Aldrich) in phosphate-buffered saline (PBS) (Fisher Scientific) was prepared at a concentration of 128 mg/L. Within a 96-well plate, a two-fold serial dilution was performed aseptically,

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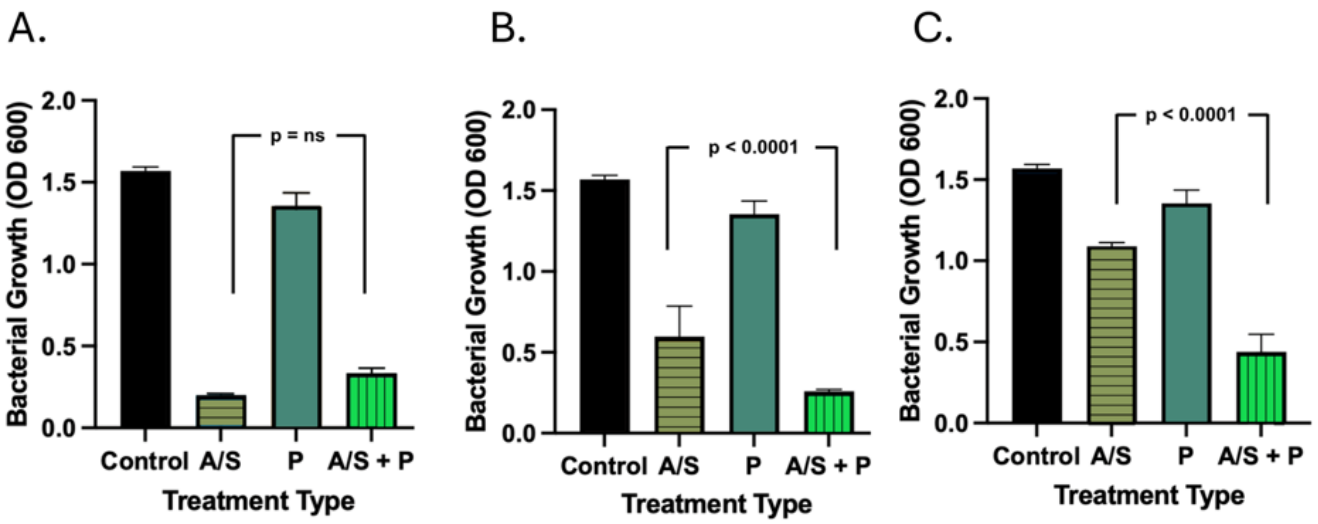

Figure 4. Piperine and ampicillin-sulbactam significantly inhibited E. coli strain E (JM109) compared to ampicillin-sulbactam alone at lower drug concentrations of 8 mg/L (Panel A), 4 mg/L (Panel B) and 2 mg/L (Panel C). Bar graphs comparing bacterial growth for the four treatment groups at varying ampicillin-sulbactam concentrations (for all graphs, n = 16 (control), n = 3 (A/S, A/S + P), n = 24 (P)). Strain E was exposed to just antibiotics, piperine, and a combination treatment with varying drug concentrations. Optical density was measured after 24h. One-way ANOVA with Tukey HSD statistical analysis between antibiotics alone and the combination treatment is indicated.

ranging from 64 mg/L to 0.5 mg/L. After 24 hours in an incubator at static growth conditions for *A. baumannii* (37 ℃), bacterial growth in the microplate (BMG Fluostar) was measured using OD 600 readings from a spectrophotometer.

Bacterial Growth in the Presence of Piperine and Ampicillin-Sulbactam.

Bacterial solutions were prepared in BHI broth as stated and adjusted to an OD 600 of 0.08 – 0.10. BHI broth was used instead of Mueller Hinton broth due to its availability. All other Clinical and Laboratory Standard Institute (CLSI) practices were implemented, including twofold dilutions and incubation time. Each drug concentration and culture condition were compared internally to the untreated controls within each experiment using the same media. Piperine was dissolved in water to create a saturated solution with a 40 mg/L concentration. A twofold serial dilution was initiated with a 128 mg/L stock solution of ampicillin-sulbactam alone or separately in the presence of 4 mg/L piperine final concentration. All treatment concentrations were completed in triplicate wells. Separately, 4 mg/L piperine without ampicillin-sulbactam was tested as a negative control for the direct antimicrobial activity of piperine. After 24 hours in an incubator at standard growth conditions (37 ℃), data for bacterial growth in the microplate was collected using OD 600 measurements.

Intracellular Antimicrobial Activity of Piperine and Ampicillin-Sulbactam

Cells from the murine macrophage RAW 264 cell line (ATTC) were counted to determine the initial cell density to seed tissue culture plates for infection with bacteria. The RAW cells were cultured in RPMI 1640 media (Invitrogen) for five days and then trypsinized. Cells were quantified using a hemocytometer. Bacteria were imaged via fluorescent excitation of DAPI with a 405 nm excitation and 460 nm emission and analyzed using the ImageJ software. All cells in a row of four boxes within the grid were counted and then multiplied by 1.6 x 10^5 to account for the entire solution. This gave us the number of macrophages per mL. The desired concentration of cells was 1.0 x 10^6 cells per well. Bacterial colonies were collected from a petri dish via a sterile loop and then submerged in 20 mL of PBS in a microcentrifuge tube. This formed the master stock for each strain used in experimentation. Each strain's stock concentration was measured using a spectrophotometer (OD 600) after 10 µL of bacterial stock was combined with 990 µL of PBS. Optical density values were used to adjust stock concentrations to 2.0 x 10^8 CFU/mL using empirically derived conversion values through direct plating and enumeration of CFU (data not shown). The cells were then pipetted into a microplate for infection, then 10 µL of bacteria was added to each well and swirled before the plates were centrifuged at 10 x g for 10 minutes at 4 ℃.

The plates were then incubated at 37 ℃ for 30 minutes. The infected RAW cells were washed in PBS before the four treatment groups were added to the wells. Experimentally infected macrophage groups were 1) infected and untreated, 2) ampicillin-sulbactam treated at concentrations of either 64 mg/L or 32 mg/L and 3) ampicillin-sulbactam treated plus piperine. The microplates were transferred to the incubator at 37 ℃, and after 12 hours, a microscope was used to count the bacteria and determine the number of bacteria present within each macrophage. Data was then analyzed by comparing the four treatment groups through a one-way ANOVA test. In addition, the post-hoc Tukey Honestly Significant Difference (HSD) test was used to compare the significance of the difference between pairs

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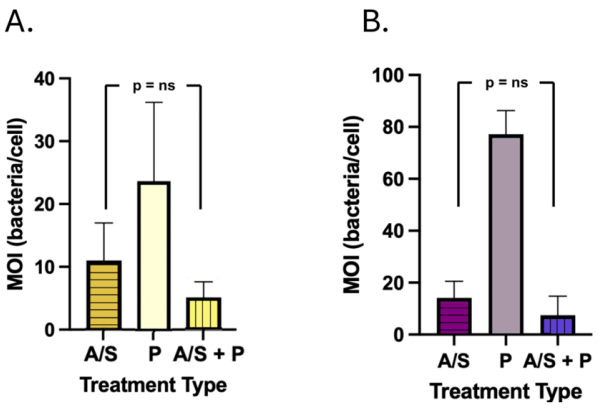

Figure 5. Piperine and ampicillin-sulbactam had a visible but insignificant impact on bacterial multiplicity of infection in RAW cells for A. baumannii strains A (H72721) (Panel A) and B (BC-5) (Panel B). Bar graphs comparing the multiplicity of infection for three treatment groups at varying ampicillin-sulbactam concentrations (for all graphs, n = 9) for two A. baumannii strains. RAW cells infected with strains A and B were then exposed to antibiotics, piperine, and a combination treatment with varying drug concentrations. Cells were counted after 12h. One-way ANOVA with Tukey HSD, $p > 0.05$ (strain A), $p > 0.05$ (strain B) between antibiotic alone and the combination treatment.

within the treatment groups. The significance of the difference between bacterial growth when exposed to just ampicillin-sulbactam and when exposed to the combination treatment was calculated for each combination of drug concentration and bacterial strain.

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