 doi:10.20944/preprints202209.0143.v1

Cannabis sativa CBD Extract Exhibits Synergy with Broad-Spectrum Antibiotics against *Salmonella typhimurium*

Logan Gildea[1], Joseph Atia Ayariga[2]*, Junhuan Xu[3], Robert Villafane[1], Boakai K. Robertson[1], Michelle Samuel-Foo[3], Olufemi S. Ajayi[3]*

[1] The Microbiology Program, College of Science, Technology, Engineering, and Mathematics (C-STEM), Alabama State University, Montgomery, AL 36104, USA; lgildea5810@myasu.alasu.edu (L.G.); rvillafane@alasu.edu (R.V.), brobertson@alasu.edu, (BKR)

[2] The Biomedical Engineering Program, College of Science, Technology, Engineering, and Mathematics (C-STEM), Alabama State University, Montgomery, AL 36104, USA

[3] Department of Biological Sciences, Alabama State University, Montgomery, AL 36104, USA; jxu@alasu.edu (J.X.); mfoo@alasu.edu (M.S.-F.); oajayi@alasu.edu (O.S.A.)

* Correspondence: *ayarigajosephatia@yahoo.co.uk (J.A.A.); oajayi@alasu.edu (O.S.A.)*

Abstract: New generation antibiotics are needed to combat the development of resistance to antimicrobials. One of the most promising new classes of antibiotics is cannabidiol (CBD). It is a non-toxic and low-resistance chemical that can be used to treat bacterial infections. The antibacterial activity of *Cannabis sativa* L. byproducts, specifically CBD, has been of growing interest in the field of novel therapeutics. As research continues to define and characterize the antibacterial activity that CBD possesses against a wide variety of bacterial species it is important to examine potential interaction between CBD and common therapeutics such as broad-spectrum antibiotics. Here, we show that CBD-antibiotic co-therapy can effectively fight *S. typhimurium* via membrane integrity disruption. This research serves to examine the potential synergy between CBD and three broad-spectrum antibiotics for potential antibiotic-CBD co-therapy. In this study, we reveal that *Salmonella typhimurium* (*S. typhimurium*) growth is inhibited at very low dosages of CBD-antibiotic. This interesting finding demonstrates that CBD and CBD-antibiotic co-therapies are viable novel alternatives to combating *Salmonella typhimurium*.

Keywords: *Salmonella*, novel antibacterial agents, cannabidiol, co-therapy, bacterial genetics

1. Introduction

Cannabis sativa L. historically has been utilized in a multitude of applications ranging from textile production to therapeutic treatment **[1-4]**. As we move towards the mid-21st century, public acceptance of *C. sativa* derived products continues to increase **[5]**. Thisgrowth in public acceptance entices exploration of applications for *C. sativa* and its by-products. Currently *C. sativa* and its byproducts have been shown to be efficacious against several neurological diseases including Tourette's syndrome, epilepsy, and multiple sclerosis **[6-8]**. The characterization of *C. sativa's* two active compounds tetrahydrocannabinol (THC) and cannabidiol (CBD) has significantly progressed our understanding of the physiological and psychological effects of *C. sativa* on humans **[9-12]**.

A topic of growing interest is the antibacterial activity of CBD against several pathogenic bacteria. Recent studies have observed CBD's antibacterial activity against several clinically relevant pathogens including *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Clostridium difficile*, *Neisseria* spp., *Moraxella catarrhalis*, *Legionella pneumophila*, and *Salmonella* spp. **[13-17]**. With the increased prevalence and occurrence of antimicrobial resistance posing a significant threat to public health, the development of novel antibacterial agents is a necessity **[18-21]**. The current rise in antibiotic and multidrug resistant bacteria is a major crisis resulting in 2.8 million infections and 35,000 deaths annually in the United State alone **[22]**. In addition, resistant bacteria present a major economic burden of approximately 7.7 billion dollars annually in the United States because of prolonged hospitalizations and exhaustion of treatment avenues **[23]**. The development of novel therapeutic agents, such as CBD, which represents a new and novel class of potential antibiotics with a unique chemical structure and low detectable resistance, is crucial in the mitigation of the crisis that resistant pathogens are responsible for.

While many notable resistant bacteria are results of hospital-acquired infections, the threat of resistant foodborne bacteria is a significant threat. *Salmonella* species are one of the most common and prevalent foodborne pathogens

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 September 2022 doi:10.20944/preprints202209.0143.v1

worldwide, found in several food products including poultry, seafood, and other fresh or processed meats **[15,21,30-33]**. Accounting for 1.35 million infections, 26,500 hospitalizations, and 420 deaths annually in the United States alone, *Salmonella* species have a significant impact on public health **[22]**. The mass and often inappropriate use of antibiotics, especially in the food industry, is a major concern and could lead to mass exposure of the public to antibiotic and multi-drug resistance pathogens **[15,18-21,34,35]**. *S. typhimurium*, the strain of focus in this study, is notable for its ability to develop resistance to antibiotics. According to the CDC, 59% of ampicillin-resistant *Salmonella* infections were attributed to *S. typhimurium* **[22]**. Considering this, *S. typhimurium* is a strain with relevancy to antibiotic resistance and the development of novel antibacterial agents.

While CBD has been utilized in several applications over the last several years, the antibacterial potential of CBD has been of growing interest **[13-16,24,25]**. Several studies have outlined the efficacy of CBD against several clinically relevant bacterial strains **[13-15,24]**, however, there remains questions of the mechanisms of antibacterial activity and the potential application methods of CBD. Literature has suggested that CBD's primarily fatty acid composition leads to membrane disruption in bacterial species, particularly gram-negative species **[24]**. While CBD has been shown to be effective in inducing bacterial death, little is known about the possibility of CBD-antibiotics co-therapy for bacterial infections. The mechanisms and influences of this interaction require further definition, but there are still several other aspects of CBD that must be investigated for the development of its use as a potential therapeutic agent. An interesting aspect of therapeutic design is the utilization of co-therapies whose efficacy is conferred through synergistic relationships between two or more therapeutic agents **[26-29]**. Determining if novel therapeutic agents, such as CBD, exhibit synergistic relationships with other therapeutic agents could further advance the development of CBD as a novel antibacterial. Considering this our study seeks to explore the effectiveness of CBD-broad spectrum antibiotic co-therapies *in vitro* against *Salmonella typhimurium*, a clinically relevant gram-negative bacterium.

Our study has utilized fluorescence microscopy, biological assays, and bacterial growth kinetics to determine the effectiveness of CBD-broad spectrum antibiotic co-therapy against *S. typhimurium*. Previous studies from our group have explored the effectiveness of *C. sativa* CBD extracts against *Salmonella* species, in this study we further explore the activity of this agent for co-therapy application. Using several biological assays for synergy we observed interactions between CBD and three broad spectrum antibiotics: ampicillin, kanamycin, and polymyxin B. The results of this study confirmed that CBD exhibits a synergetic relationship with these broad-spectrum antibiotics, particularly polymyxin B. Extended kinetics also confirmed that co-therapy treatments did not result in developed resistance over the span of 24 hours. The results of these studies provide new insight into the potential of CBD in co-therapy applications thus progressing the research and development of CBD as a potential antibacterial agent.

2. Materials and Methods

2.1. CBD Extraction and Preparation

CBD extraction was carried out by Sustainable CBD LLC. *C. sativa* biomass was weighed, tagged, and recorded in a receiving trailer for processing. Following storage, the biomass was reduced to between 200 and 500 microns and underwent CO_2 extraction in an Apeks Transformer for subcritical extraction (Gibraltar Industries Inc., Buffalo, NY, USA). Subcritical extraction was carried out at a target pressure of 1200 psi, chiller temperature of 20–25 °C, propylene glycol percentage of 10%, orifice size of 22, resultant separator pressure of 350–400 psi, resultant separator temperature of −6–4 °C, for an extraction time of approximately 2–3 h. Following subcritical extraction, samples were prepared for decarboxylation prior to supercritical extraction. Hemp biomass was placed in the oven for approximately 100 min at 265 °C to decarboxylate. Once decarboxylation was carried out, an Apeks Transformer was utilized for supercritical extraction (Gibraltar Industries Inc., Buffalo, NY, USA). Supercritical extraction was carried out at a target pressure of 1800 psi, chiller temperature of 37–42 °C, propylene glycol percentage of 10%, orifice size of 18, resultant separator pressure of 350–400 psi, resultant separator temperature of 0–10 °C, for an extraction time of approximately 1–2 h per pound of material. The resulting material then underwent winterization through addition of ethanol to the crude extract. This sample was frozen and then filtered through Buchner funnels and the remaining ethanol was evaporated using a Heidolph rotary evaporator (Heidolph Instruments GmbH & Co.KG, Kelheim, Germany). Distillation was carried out using the Lab Society 5 L short path distillation unit (Lab Society®, Boulder, CO, USA) for further refinement. The resulting product of this procedure was winterized cannabinoid **[36]. The** Winterized CBD crude oil obtained from

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 September 2022 doi:10.20944/preprints202209.0143.v1

Sustainable CBD LLC. (Salem, AL, USA) was diluted with 4% EtOH and vortexed to a final concentration of 50 mg/mL. From this stock serial dilutions were utilized to create our experimental concentrations of 1 µg/mL, 0.1 µg/mL, 0.01 µg/mL, or 0.001 µg/mL. CBD variety 'Suver Haze' (Z3H1-0101-24521-13522-13622) was utilized for these studies.

2.2. Bacterial Growth Kinetics

To study bacterial growth kinetics, a 96-well plate (Fisherbrand™, Fisher Scientific, Fair Lawn, NJ, USA) was inoculated with 180µL of overnight bacterial cultures of *S. typhimurium* at an OD600 ≈ 0.15. The cultures were then incubated at 37 °C with rotary shaking at 121 rpm. Measurements of bacterial density (OD600) were taken hourly for 24 h using a spectrometer (Molecular Devices SpectraMax® ABS Plus) (Molecular Devices LLC., San Jose, CA, USA). This experiment was completed in triplicate. **[15,21]**

2.3. Bacterial Growth Kinetics in the Presence of CBD

To study how CBD affects bacterial growth kinetics, three 96-well plates were inoculated with overnight bacterial cultures of *S. typhimurium* at an OD600 ≈ 0.15. These cultures were then treated with varying concentrations of CBD (1 µg/mL, 0.1 µg/mL, 0.01 µg/mL, or 0.001 µg/mL). The cultures were then incubated at 37 °C with rotary shaking at 121 rpm. Measurements of bacterial density (OD600) were taken hourly for 24 h using a spectrometer. This experiment was completed in triplicate **[15]**.

2.4. Bacterial Growth Kinetics in the Presence of Antibiotics

To study how antibiotic affects bacterial growth kinetics, three 96-well plates were inoculated with overnight bacterial cultures of *S. typhimurium* at an OD600 ≈ 0.15. These cultures were then treated with either ampicillin, kanamycin, or polymyxin B at varying concentrations. Antibiotics were serially diluted to concentrations of 50, 5, 0.5, 0.05 µg/mL. Treated bacterial cultures were then incubated at 37 °C with rotary shaking at 121 rpm. Measurements of bacterial density (OD600) were taken hourly for 24 h using a spectrometer. This experiment was completed in triplicate **[15]**.

2.5. Comparative Bacterial Growth Kinetics in the Presence of Antibiotics or CBD

To compare the effect of antibiotics and CBD co-treatment on bacterial growth kinetics, three 96-well plates were inoculated with overnight bacterial cultures of *S. typhimurium* an OD600 ≈ 0.15. These cultures were then treated with either ampicillin (5 µg/mL), kanamycin (5 µg/mL), polymyxin B (5 µg/mL) or CBD (1 µg/mL). The cultures were then incubated at 37 °C with rotary shaking at 121 rpm. Measurements of bacterial density (OD600) were taken hourly for 24 h using a spectrometer (Molecular Devices SpectraMax® ABS Plus). This experiment was completed in triplicate **[15]**.

2.6. CBD-Antibiotic Synergy Assay

To study the potential synergy between 3 broad spectrum antibiotics (ampicillin, kanamycin, and polymyxin B) and CBD the checkerboard assay was utilized **[15,37]**. *S. typhimurium* was seeded into a 96 well plate at an OD of 0.15 (50 µL/10 mL of media). Ampicillin, kanamycin, and polymyxin B were serially diluted to concentrations of 50, 5, 0.5, and 0.05 µg/mL. CBD was serially diluted to concentrations of 1, 0.1, 0.01, and 0.001 µg/mL. Synergistic effects were determined through OD600 measures from the checkerboard assay and fractional inhibitory concentration index (FICI) following 24 h incubation at 37 °C. The FICI was calculated as below:

(1) FIC Antibiotic (^{AB}) = MIC^{AB} in Combination/ MIC^{AB} alone;
(2) FIC Cannabidiol Extract (^{CBD})=MIC^{CBD} in Combination/ MIC^{CBD} alone;
(3) $FICI = FIC^{AB} + FIC^{CBD}$

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 September 2022 doi:10.20944/preprints202209.0143.v1

The FICI was evaluated as follows: synergy (FICI < 0.5), partial synergy (0.5 ≤ FICI ≥ 0.75), additive (0.76≤ FICI ≥ 1), indifference (1 ≤ FICI ≥ 4), or antagonism (FICI > 4). The average MIC from three independent experiments were used for these equations **[37]**.

Percent inhibition calculations quantify reduction of bacterial growth 24 h following treatment utilizing control OD600 as the baseline value for inhibition. Percent inhibition was calculated as below:

(*Eq*.) % Inhibition = 100%- ((Experimental^{OD600}/Control^{OD600}) x 100%))

Experimental^{OD600} = Represents OD600 value of *S. typhimurium* treated with either: Antibiotic monotherapy, CBD monotherapy, or Antibiotic-CBD co-therapy.

Control^{OD600}= Represents OD600 value of untreated *S. typhimurium*.

Percent Inhibition was used as a comparative measure of various treatments to assess synergistic activity. The average OD600 from three independent experiments were used for these equations.

2.7 Immunofluorescent Live-Dead Assay

To examine the antibacterial activity of antibiotic-CBD cotreatment the live-dead assay was utilized with SYTO-9 (Invitrogen, Waltham, MA, US) and propidium iodine (Sigma-Aldrich, St. Louis, MO, USA) following protocols published by Ayariga et al. (2022) **[38]**. *S. typhimurium* was grown overnight then diluted to an OD600 of 0.2 using LB broth. A dH_2O treated and a 2% SDS treated *S. typhimurium* sample served as controls. Experimental samples consisted of antibiotic treated samples at concentrations of 0.5 μg/mL and 5 μg/mL, and antibiotic-CBD cotreatments with concentrations derived from synergetic potential in the checkerboard assay. Samples were incubated at 37°C for 3 h. Afterwards, samples were stained with 1 X SYTO-9 and 40 μg/mL of propidium iodine and left at room temperature for 25 min, without exposure to light. Samples were then visualized using an EVOS FLC microscope (Life Technologies).

2.8 Statistical Analysis

All experiments were performed on independent biological replicates. Statistical significance was determined for control and experimental groups using paired sample t-test. Data points were excluded if contamination was identified. Statistical analyses were preformed using Microsoft Excel (Microsoft 2010). Values reported as mean ± SE, p-values ≤ 0.05 were considered statistically significant.

3. Results and discussion

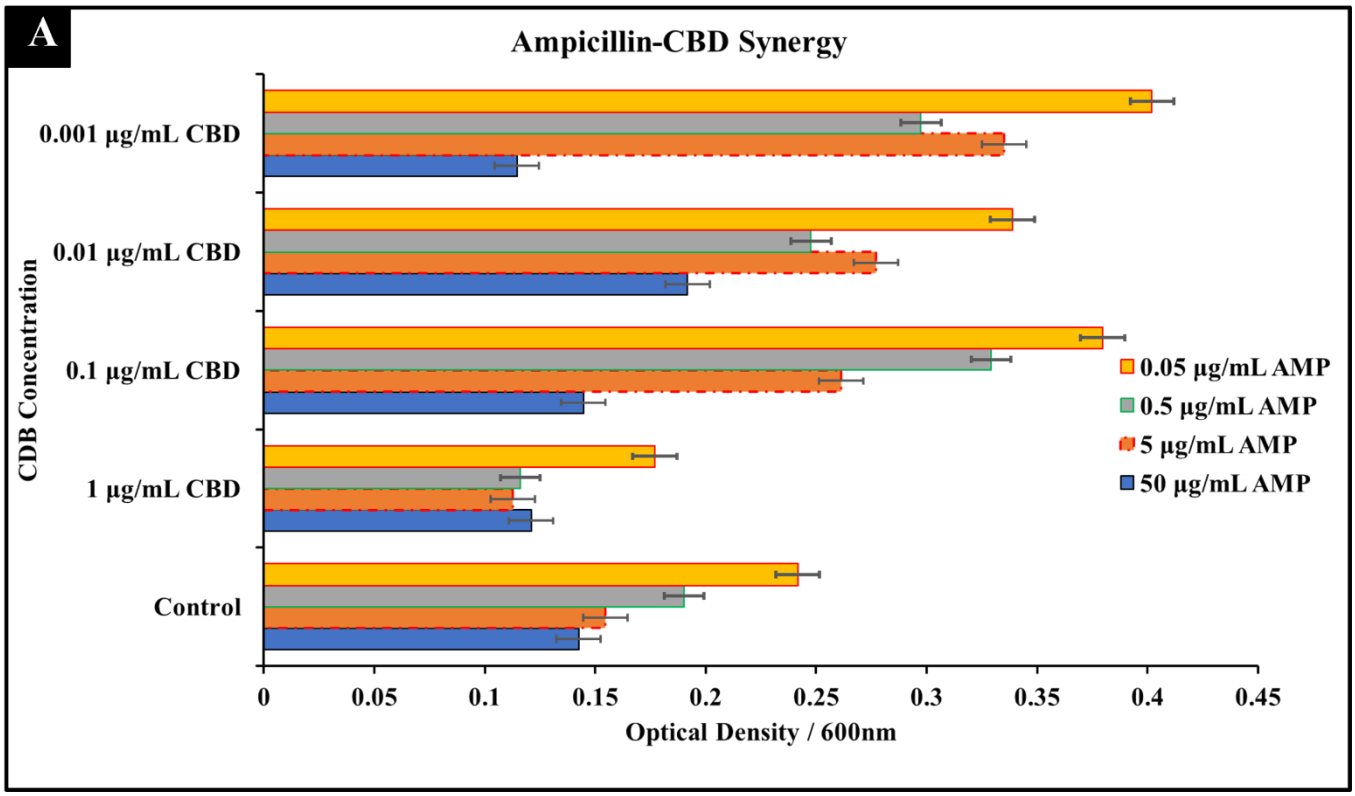
3.1 Synergetic Characteristics of CBD-Broad Spectrum Antibiotic Co-therapy

To observe the potential synergistic characteristics of CBD-antibiotic co-therapy against *S. typhimurium*, the checkerboard-synergy assay was utilized **[21]**. This method allows to observe synergy between two agents against a target bacterium through a measure of OD600 over a 24 h period. This study measured the synergy between CBD and three broad-spectrum antibiotics, ampicillin, kanamycin, and polymyxin B. *S. typhimurium* cultures were treated with antibiotic at concentrations of 50 μg/mL, 5 μg/mL, 0.5 μg/mL, or 0.05 μg/mL and CBD at concentrations of 1 μg/mL, 0.1 μg/mL, 0.01 μg/mL, or 0.001 μg/mL. For qualitative examination of the effects of antibiotic-CBD treatment on *S. typhimurium*, immunofluorescent studies were conducted. Utilizing a live/dead staining technique, the antibacterial activity of our potentially synergistic combinations was visualized comparatively to mono-treatments of ampicillin, kanamycin, and polymyxin B **(Figure 1C)**. These images further demonstrate the synergistic impact of antibiotic-CBD co-treatment and suggest that this treatment method results in greater antibacterial activity than that of antibiotic treatment alone. The images acquired through this assay further confirmed the synergistic activity between our broad-spectrum antibiotics and CBD against *S. typhimurium*. Red fluorescence, signifying cell death, is seen as the dominant color within all co-treatments suggesting the successful antibacterial activity of these combinations against our target bacterium. In comparison to combination treatment, monotreatment, emitted greater amounts of green fluorescence representative of viable living cells remaining within the culture. The results ultimately suggest that cotreatment presents significantly

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 September 2022 doi:10.20944/preprints202209.0143.v1

more antibacterial activity than observed in the mono-antibiotic treatments. The results of these assays suggest that all three antibiotics exhibit synergetic activity in combination with CBD.

In comparison to ampicillin monotherapy, it was observed that an addition of CBD at a concentration of 1 µg/mL to ampicillin at concentrations of 50 µg/mL, 5 µg/mL, 0.5 µg/mL, or 0.05 µg/mL resulted in a greater inhibition of *S. typhimurium* growth **(Figure 1)**. At concentrations of 0.5 µg/mL ampicillin and 1 µg/mL CBD in co-treatment was observed as the treatment with the highest inhibitory effect determined through comparison to ampicillin monotherapy at both the MIC concentration (5 µg/mL) and the experimental ampicillin concentration (0.5 µg/mL). CBD-Ampicillin co-treatment resulted in a lower OD600 than that of ampicillin monotherapy at the MIC concentration suggesting that co-treatment was more effective in reducing the bacterial growth over the 24 h period than the standard mono-treatment. These results suggest that ampicillin and CBD exhibit a synergetic antibacterial interaction that leads to greater inhibition than ampicillin alone **(Figure 1)**.



Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 September 2022 doi:10.20944/preprints202209.0143.v1

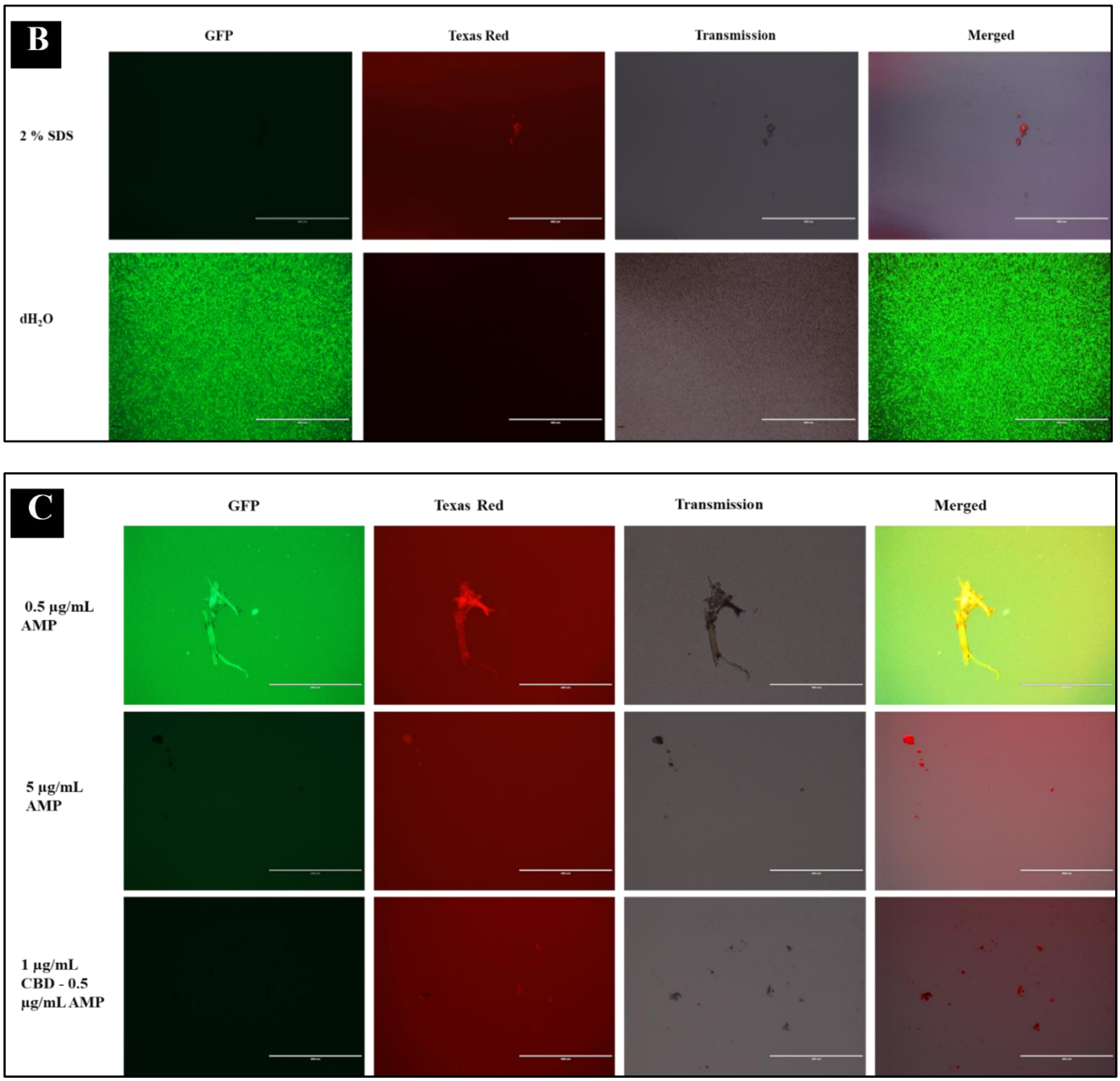
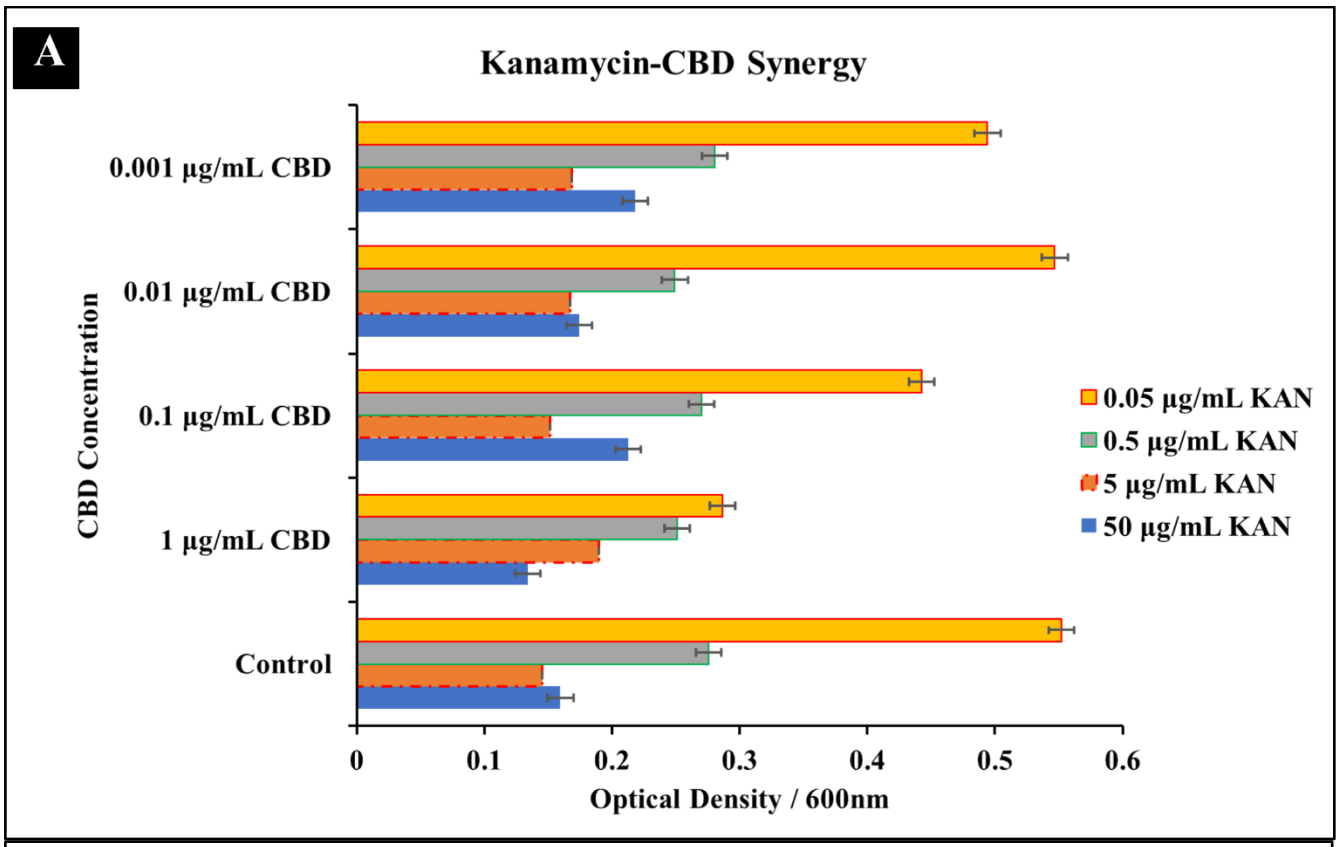
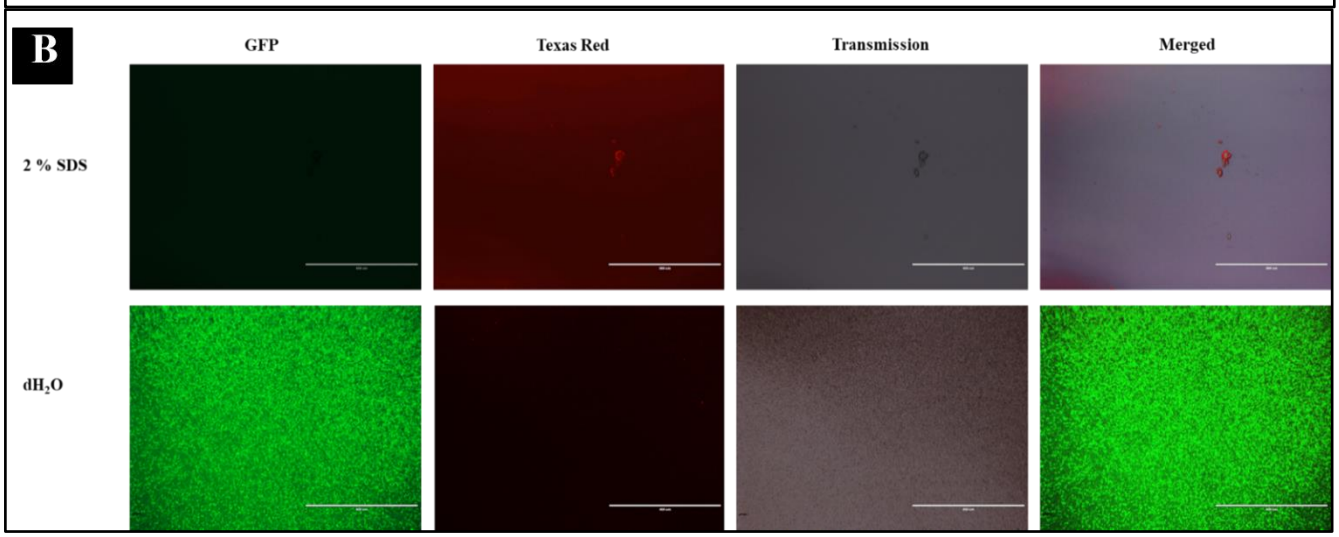


Figure 1. Synergistic analysis of ampicillin and CBD co-treatment of *S. typhimurium*. **(A)** Data presented as mean ±Standard error of mean. Synergistic activity was observed at 0.5 µg/mL ampicillin and 1 µg/mL CBD co-treatment. **(B)** Immunofluorescent staining analysis positive (2% SDS) and negative (dH_2O) controls. **(C)** Immunofluorescent staining of *S. typhimurium* treated with ampicillin at 0.5 and 5 µg/mL as well as the observed synergistic combination of 0.5 µg/mL ampicillin and 1 µg/mL CBD.

In comparison to kanamycin monotherapy, concentrations of 1 µg/mL CBD and 50 µg/mL of kanamycin in co-treatment resulted in a greater inhibition of *S. typhimurium* growth after 24 h **(Figure 2)**. These results suggest that CBD-kanamycin co-treatment results produced a greater inhibitory effect than single treatments of kanamycin at any concentration. While the inhibitory effect was increased, the effect did not indicate complete synergy. Considering this, the combination can be more accurately described as an additive. Activity between these two agents was limited at 50 µg/mL and 5 µg/mL kanamycin concentrations and there was no observed synergy at lower kanamycin concentrations. Immunofluorescent staining results reached a similar conclusion with similar effectiveness between kanamycin at the MIC concentration and kanamycin-CBD cotreatment. These results in whole suggest that kanamycin effectiveness is not impacted by the addition of CBD in both the checkerboard assay as well as the staining assay **(Figure 2C)**.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 September 2022 doi:10.20944/preprints202209.0143.v1





Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 September 2022 doi:10.20944/preprints202209.0143.v1

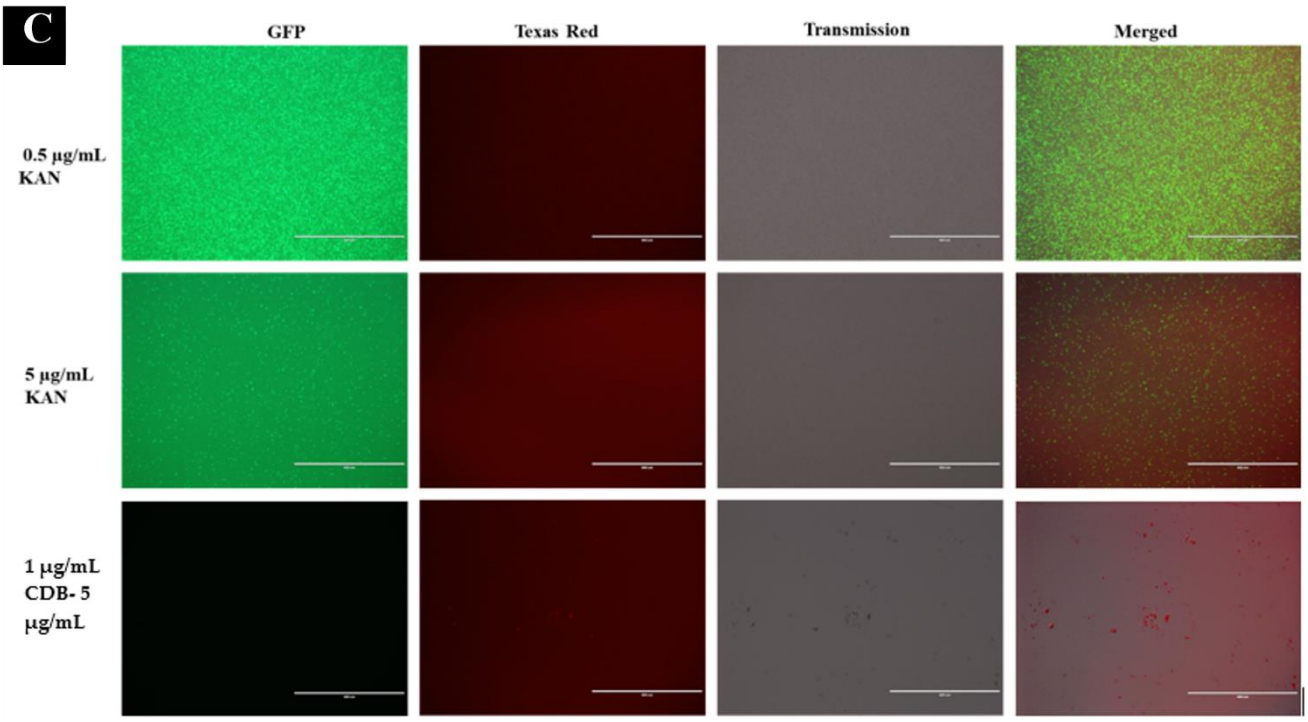
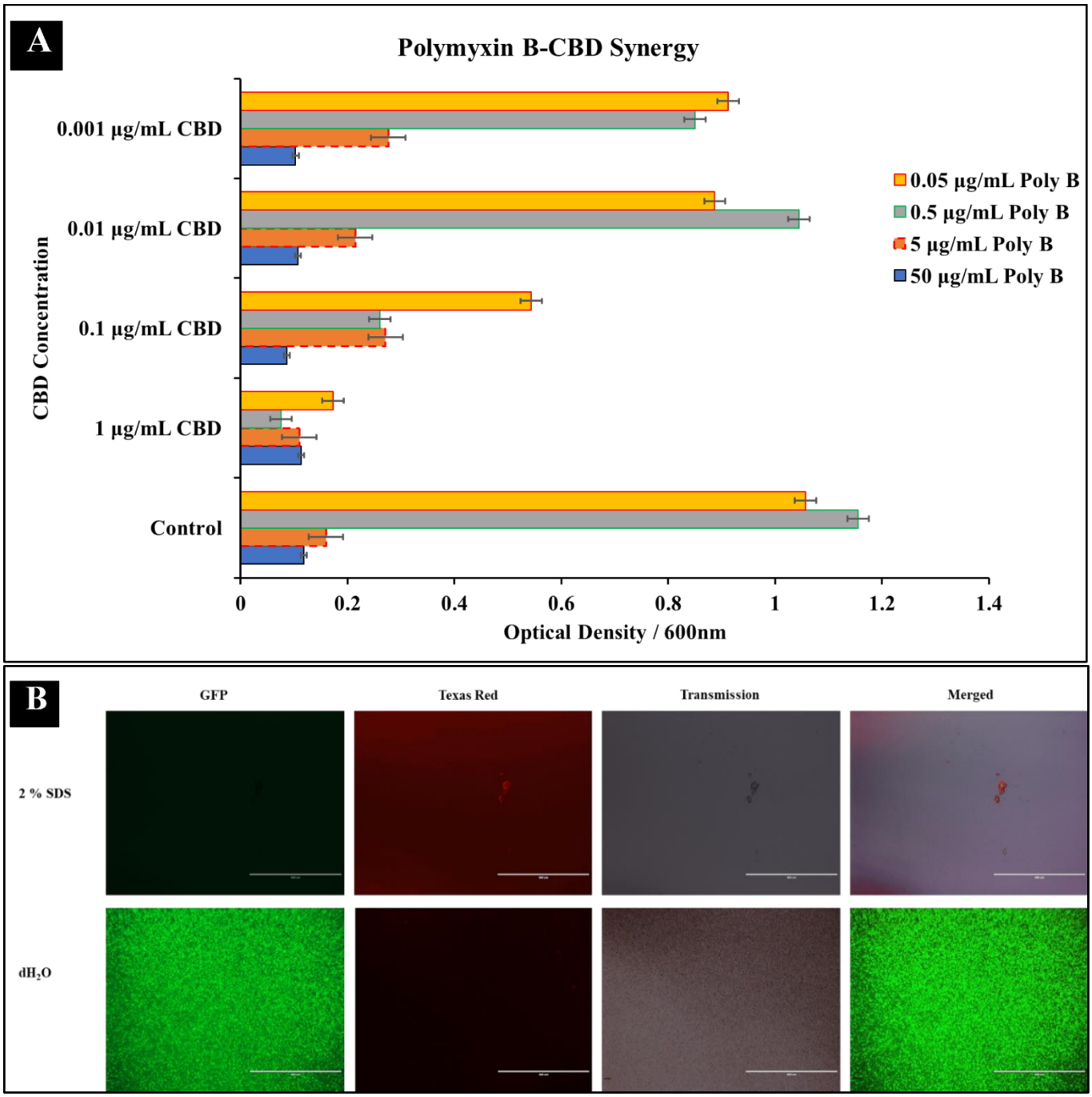


Figure 2. Synergistic analysis of kanamycin and CBD co-treatment of *S. typhimurium*. **(A)** Data presented as mean ±Standard error of mean. Potential synergistic activity was observed at 5 µg/mL kanamycin and 1 µg/mL CBD co-treatment. **(B)** Immunofluorescent staining analysis positive (2% SDS) and negative (dH_2O) controls. **(C)** Immunofluorescent staining of *S. typhimurium* treated with kanamycin at 0.5 and 5 µg/mL as well as the observed synergistic combination of 5 µg/mL kanamycin and 1 µg/mL CBD.

The final antibiotic that exhibited promising antibacterial synergy with CBD was polymyxin B. Results from both the checkerboard assay as well as immunofluorescent staining suggest that these two agents exhibit a synergetic relationship **(Figure 3)**. Polymyxin B monotherapy at a MIC of 5 µg/mL exhibited a percent inhibition of 83%, whereas co-treatment with Polymyxin at 0.5 µg/mL and CBD at 1 µg/mL resulted in a percent inhibition of 92% **(Figure 4)**. These results suggest that addition of CBD to polymyxin B can significantly reduce the required MIC while still retaining equivalent antibacterial activity. Immunofluorescent staining further confirmed the antibacterial activity that polymyxin B and CBD exhibit in treatment **(Figure 3C)**. While co-treatment with polymyxin B and CBD resulted in a significant increase in inhibition, results from the checkerboard assay also exhibit antagonism at various concentrations. Antagonism can be observed in lower CBD concentrations at the MIC Polymyxin B concentration **(Figure 3A**). Results suggest that lower concentrations of CBD in combination with polymyxin B may partially inhibit the success of the antibiotic ability to kill the bacterial. While this antagonism is important to note, it does not discredit the synergy that polymyxin B and CBD possesses at other concentrations.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 September 2022 doi:10.20944/preprints202209.0143.v1



Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 September 2022 doi:10.20944/preprints202209.0143.v1

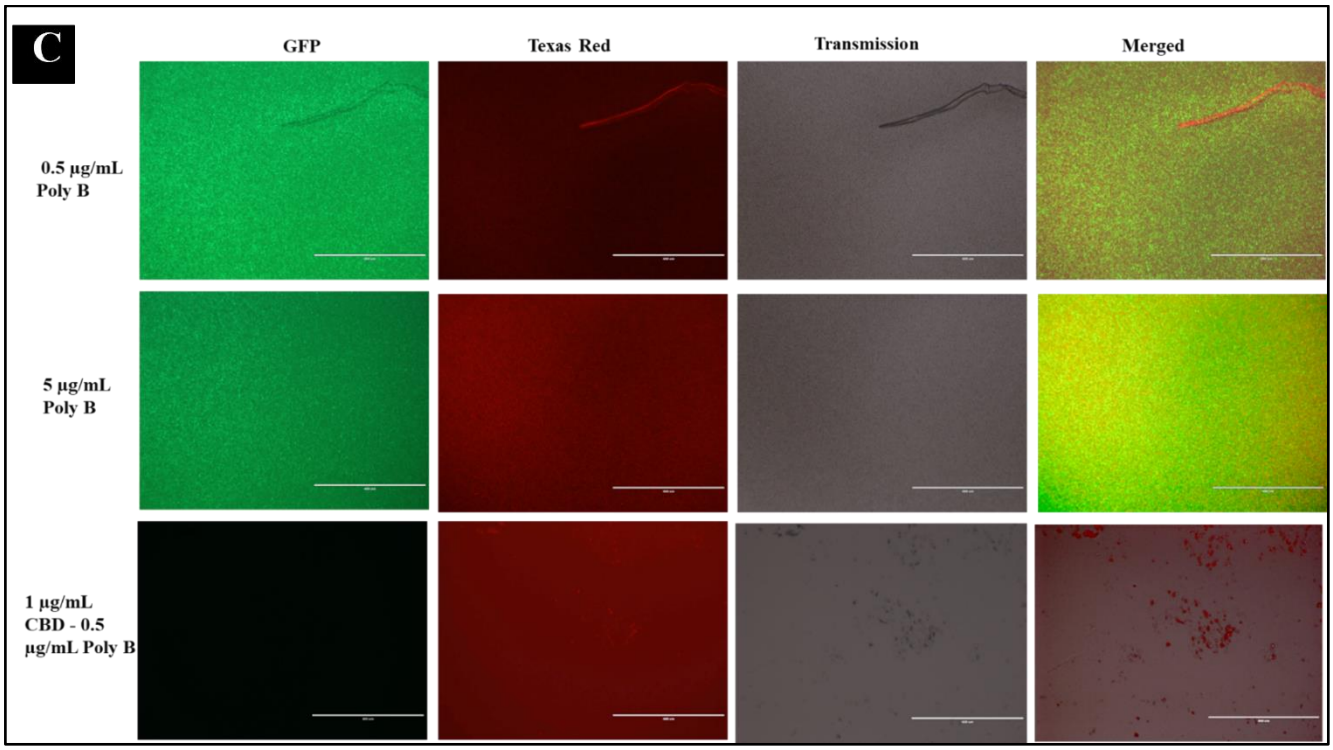


Figure 3. Synergistic analysis of kanamycin and CBD co-treatment of *S. typhimurium*. **(A)** Data presented as mean ±Standard error of mean. Potential synergistic activity was observed at 0.5 µg/mL polymyxin B and 1 µg/mL CBD co-treatment. **(B)** Immunofluorescent staining analysis positive (2% SDS) and negative (dH_2O) controls. **(C)** Immunofluorescent staining of *S. typhimurium* treated with polymyxin B at 0.5 and 5 µg/mL as well as the observed synergistic combination of 0.5 µg/mL polymyxin B and 1 µg/mL CBD.

To further quantify the effectiveness of antibiotic-CBD cotreatment and to compare monotherapy to co-therapy, percent inhibition was calculated from the checkerboard assay **(Figure 4)**. Percent inhibition serves to comparatively assess OD600 between treated samples with untreated samples serving as the baseline. These measures further confirmed the synergy exhibited between both ampicillin and polymyxin B, with CBD. Additionally, this measure further suggests the additive or indifferent activity between kanamycin and CBD. We observe significant increases in percent inhibition in both polymyxin B and ampicillin when in cotreatment with CBD further suggesting the synergetic activity between these agents. It is observed that this cotreatment strategy significantly increases the effectiveness of CBD, polymyxin B, and ampicillin against *S. typhimurium* in comparison to monotreatment.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 September 2022 doi:10.20944/preprints202209.0143.v1

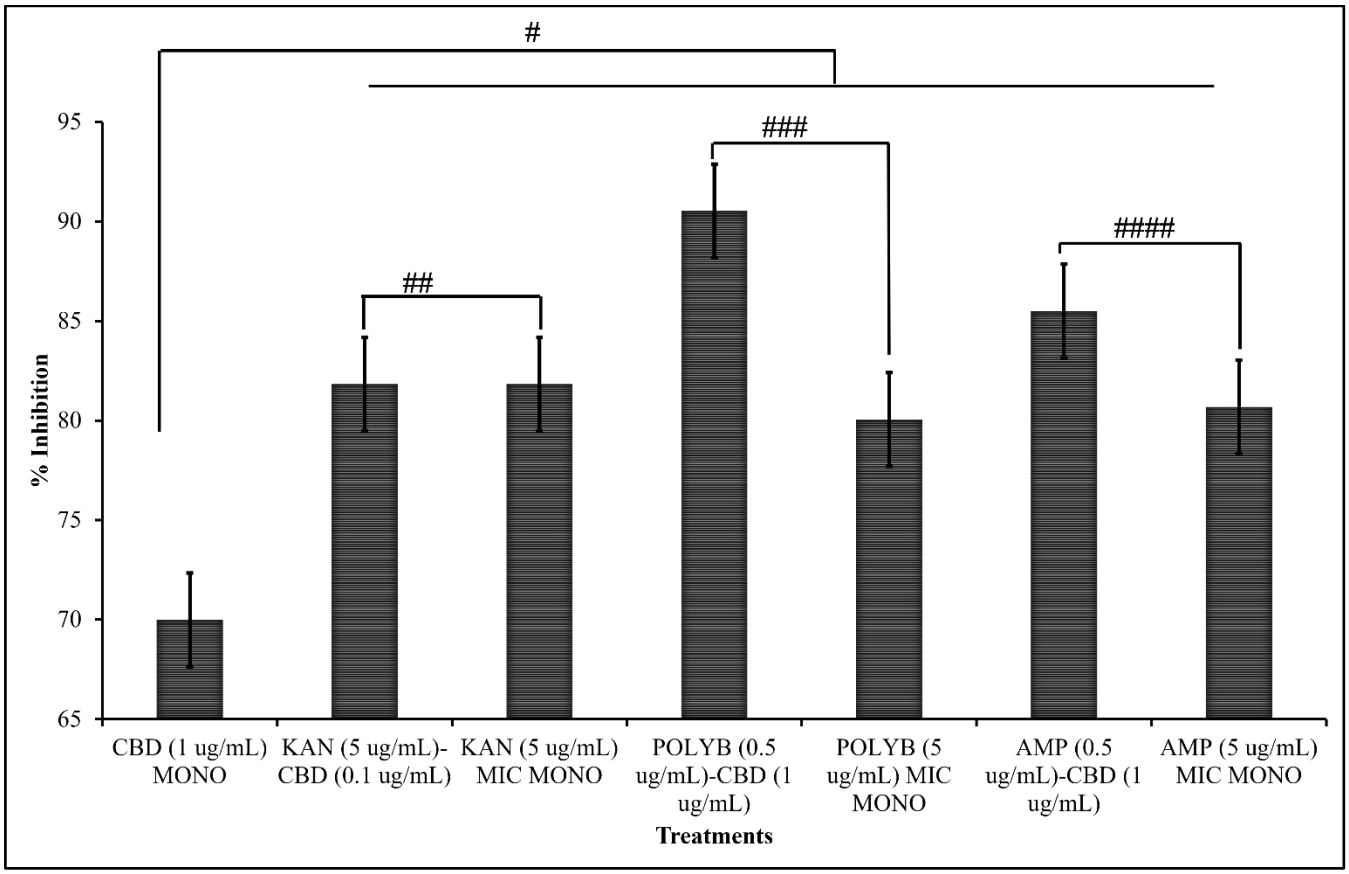
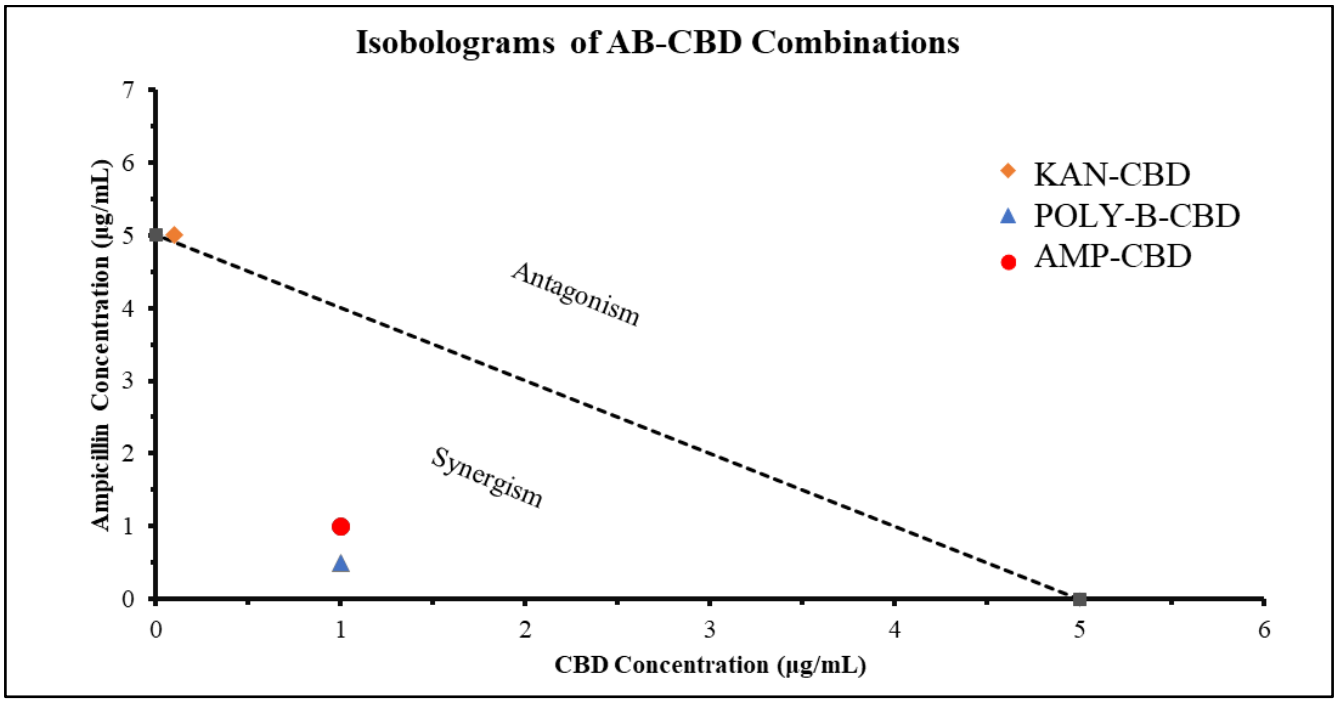


Figure 4. Percent Inhibition of both monotreatment at MIC and cotreatment at synergistic concentrations as determined through checkerboard screening. (SEM # = 0.059, ## = 0.018, ### = 0.006, #### = 0.062).

3.1.2 Synergistic Interaction Analysis

To further confirm the results obtained through checkerboard assay **(Figure 1-4)**, synergistic effects were further quantified through FICI. The FICI was calculated for the three combinations that presented potentially synergetic effects within the checkerboard assays **(Figure 5)**. The FICI results quantified what was observed through the checkerboard assays and qualified CBD-Ampicillin and CBD-Polymyxin B as having synergistic activity against *S. typhimurium*. FICI calculations determined that the relationship between CBD-Kanamycin could be described most accurately as indifference, or a potential additive **(Figure 5)**.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 September 2022 doi:10.20944/preprints202209.0143.v1



	Antibiotic		*Cannabidiol*			
Combination	**MIC Alone**	**MIC Combination**	**MIC Alone**	**MIC Combination**	**FICI**	**Interpretation**
Ampicillin + CBD	5 µg/mL	0.5 µg/mL	5 µg/mL	1 µg/mL	0.3	Synergy
Kanamycin + CBD	5 µg/mL	5 µg/mL	5 µg/mL	0.1 µg/mL	1.02	Indifference
Polymyxin B + CBD	5 µg/mL	0.5 µg/mL	5 µg/mL	1 µg/mL	0.3	Synergy

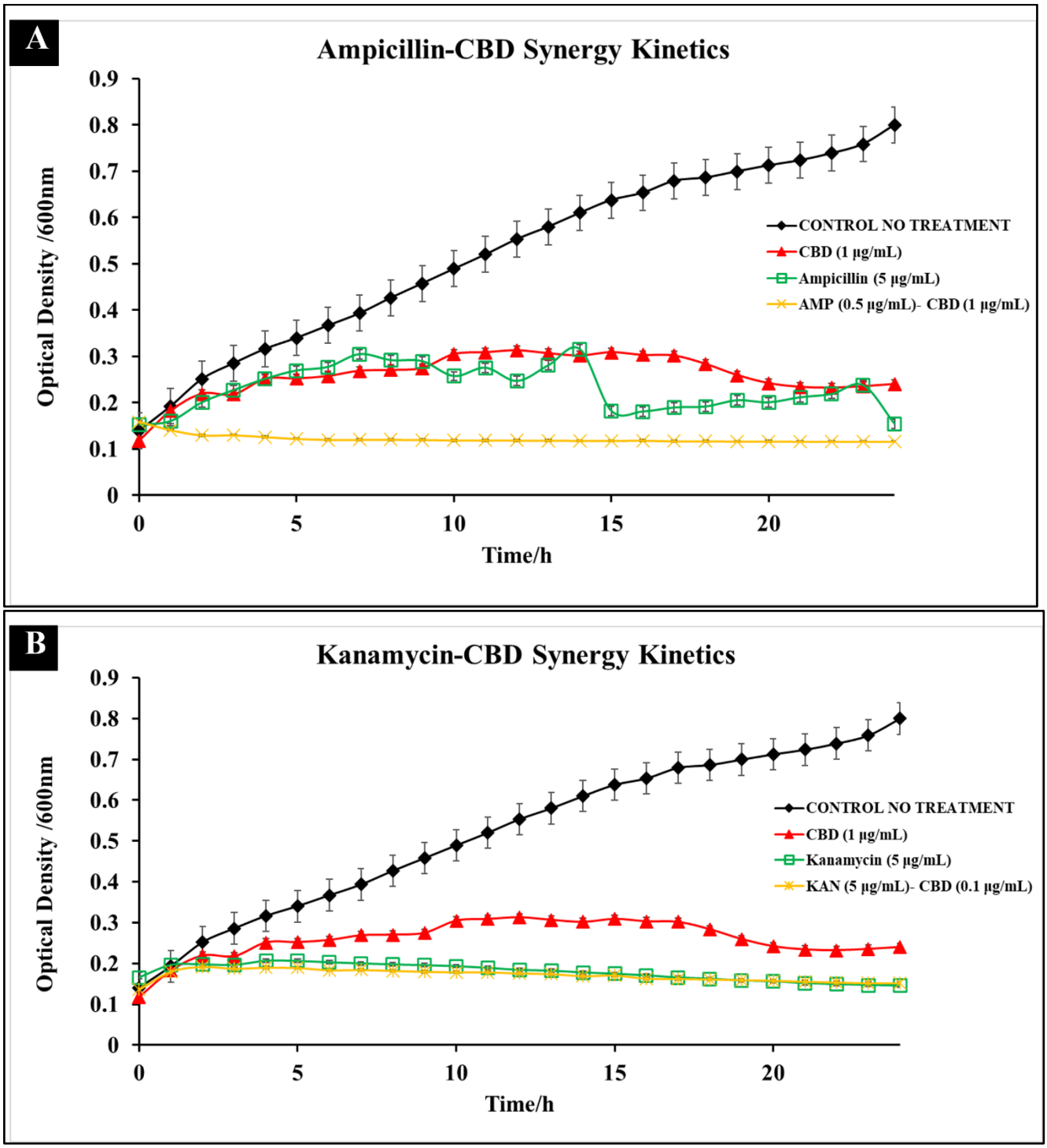
Figure 5. Synergistic activity of CBD and Antibiotics (Ampicillin (AMP), Kanamycin (KAN), Polymyxin-B (POLY-B)) against *S. typhimurium*. Isobologram showing the synergy (FICI < 0.5), partial synergy (0.5 ≤ FICI ≥ 0.75), additive (0.76≤ FICI ≥ 1), indifference (1 ≤ FICI ≥ 4), or antagonism (FICI > 4) effects of CBD paired with broad-spectrum antibiotics against *S. typhimurium*. Representative data for determination of FICI is included.

3.2 Comparative Kinetics of antibiotic-CBD Co-treatment

Broad spectrum antibiotics have been widely used as a common treatment method against *Salmonella*. To compare the efficacy of antibiotic monotherapy and antibiotic-CBD co-treatment bacterial growth kinetics were recorded over the span of 24 h. These studies serve to further define and characterize the synergetic antibacterial activity that these antibiotic-CBD combinations possess against *S. typhimurium*. The impact on *S. typhimurium* growth was compared between antibiotic monotherapy, CBD monotherapy, and CBD-antibiotic co-treatment.

The three co-treatments examined were selected based on the synergistic activity observed in the checkerboard assays. The three co-treatments examined were ampicillin (0.5 µg/mL)-CBD (1 µg/mL), kanamycin (50 µg/mL)-CBD (1 µg/mL), and polymyxin B (0.5 µg/mL)-CBD (1 µg/mL). We observed that all co-treatments reduced the OD600 of *S. typhimurium* greater than monotherapies at MIC concentrations over the 24 h period **(Figure 6)**. We observe the highest inhibitory effects in ampicillin-CBD **(Figure 6A)** and polymyxin B-CBD **(Figure 6C)** whereas in kanamycin the effect of treatment is relatively similar between kanamycin-monotherapy and kanamycin-CBD co-treatment **(Figure 6B)**.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 September 2022 doi:10.20944/preprints202209.0143.v1



Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 September 2022 doi:10.20944/preprints202209.0143.v1

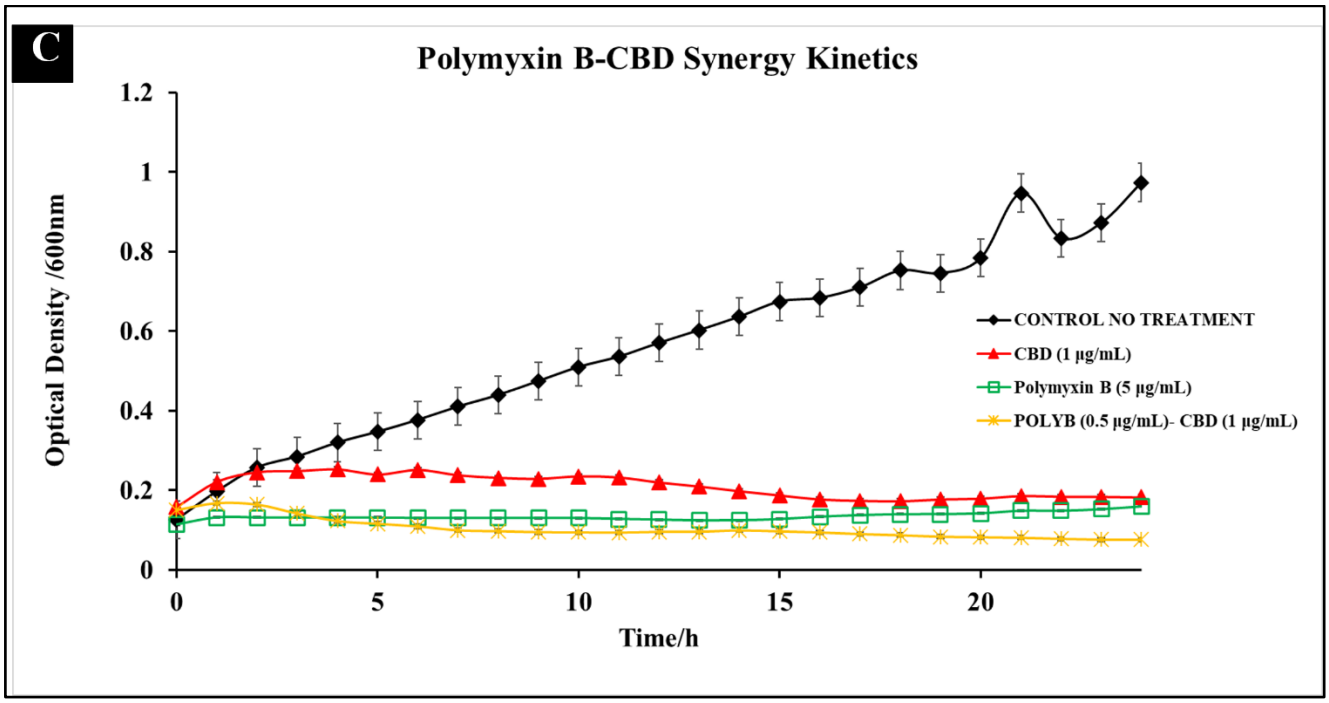


Figure 6. Comparative efficacy of CBD-antibiotic co-treatment and MIC antibiotic monotherapy against *S. typhimurium*. Three co-treatments examined include ampicillin (0.5 µg/mL)-CBD (1 µg/mL) **(A)**, kanamycin (50 µg/mL)-CBD (1 µg/mL) **(B)**, and polymyxin B (0.5 µg/mL)-CBD (1 µg/mL) **(C)**.

The results of these comparative kinetics assays showed that 1) Co-treatment was more effective than the MIC concentration in a monotherapy application in ampicillin and polymyxin-B and 2) Co-treatment resulted in little resistance development over a 24 h period. *S. typhimurium's* ability to rapidly develop resistance has been a considerable concern to public health **[22]**. In previous studies it was shown that *S. typhimurium* was able to develop resistance against CBD treatment 24 h after treatment **[15]**. For this reason, it is significant to note that in CBD-antibiotic co-treatments, there were limited resistance development over the 24 h period suggesting that co-therapy could potentially reduce the ability of *S. typhimurium* to develop resistance.

These results further confirm the potential effectiveness of co-treatment with CBD. In these studies, we observe that antibiotic concentrations lower than the MIC remain effective in reducing bacterial growth when paired with CBD as a synergistic additive. This effectiveness is clearly depicted particularly in ampicillin **(Figure 6A)** and polymyxin-B **(Figure 6C)** where we observe co-treatment resulted in significantly lower OD600s 24 hours after treatment in comparison to antibiotics at MIC values. This was as expected, as we observed the most synergistic activity between polymyxin-B and ampicillin, with CBD. In kanamycin, we observe a negligible difference in OD600 other the 24 h period **(Figure 6B)**. This result correlated with the lack of synergy observed within the checkerboard assay and calculated through the FICI **(Figure 5)**.

In this time of antibiotic resistance, it is crucial to develop new antibacterial agents as well as develop alternative treatment methods against these resistant bacteria. The lack of antibiotic development in the 21st century has enhanced the need for researchers to repurpose antibiotics and determine treatment methods that help retain their antibacterial function. The use of agents extracted from botanical sources as synergetic additives has been an area of growing interest over recent history **[15,37,39,40]**. This study explored the potential synergistic activity between *C. sativa* L. CBD extract and three broad spectrum antibiotics: ampicillin, kanamycin, and polymyxin-B. The results of these studies suggest that CBD does exhibit synergetic activity with both ampicillin and polymyxin-B against *S. typhimurium*, providing a potentially effective cotreatment method. Further study of cytotoxic effects, potential delivery mechanisms, and the effect of CBD treatment on *S. typhimurium* gene expression are all necessary to further progress the development of this potential co-treatment method.

4. Conclusion

Antibiotic resistant bacterial infections have been a significant hazard to world health over the 21st century. The lack of antibiotic development has increased the need for new antibacterial agents and treatment strategies. In this study we

examined the potential synergy between three broad-spectrum antibiotics and *C. sativa* L. CBD extract against *S. typhimurium*, a prevalent and hazardous foodborne pathogen. The results of our study confirmed that CBD does exhibit synergistic activity with both ampicillin and polymyxin-B against *S. typhimurium*. The results suggest that CBD-antibiotic co-therapies could have potential promise as novel therapeutic options.

Author Contributions: Conceptualization, LG and JAA; methodology, LG and JAA; software, LG and JAA; validation, OA, BK, and MS; formal analysis, LG, JAA, JX, BK, MS and OA; investigation, LG, JAA, JX, BK, MS, and OA; resources, JAA, RV, MS, and OA; data curation, LG, JAA, JX, MS, and OA.; writing—original draft preparation, LG; writing—review and editing, LG and JAA; visualization, LG and JAA.; supervision, JAA, BK and OA; project administration, OA; funding acquisition, OA. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by United States Department of Education, Title III- HBGI-RES, and National Science Foundation; and HBCU-RISE; grant number: 1646729.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article.

Acknowledgments: The authors acknowledge Professor Derrick Dean, Professor Vida Dennis for their HBCU-RISE grant support. We also acknowledge Alabama State University, C-STEM for supplies and Laboratory space. The authors acknowledge receiving funding from United States Department of Education, Title III- HBGI-RES.

Conflicts of Interest: The authors declare no conflict of interest.

Sample Availability: Samples of the compounds are available from the authors.

Reference

1. Long, T., Wagner, M., Demske, D. et al. Cannabis in Eurasia: origin of human use and Bronze Age trans-continental connections. Veget Hist Archaeobot 26, 245–258 (2017). https://doi.org/10.1007/s00334-016-0579-6
2. Zuardi, A.W. (2006) History of cannabis as a medicine: a review. Rev Bras Psiquiatr. 28:153-157.
3. Crini, G., Lichtfouse, E., Chanet, G. et al. Applications of hemp in textiles, paper industry, insulation and building materials, horticulture, animal nutrition, food and beverages, nutraceuticals, cosmetics and hygiene, medicine, agrochemistry, energy production and environment: a review. Environ Chem Lett 18, 1451–1476 (2020). https://doi.org/10.1007/s10311-020-01029-2
4. Rehman, M., Fahad, S., Du, G. et al. Evaluation of hemp (Cannabis sativa L.) as an industrial crop: a review. Environ Sci Pollut Res 28, 52832–52843 (2021). https://doi.org/10.1007/s11356-021-16264-5
5. Peters, J. and Chien, J. "Contemporary Routes of Cannabis Consumption: A Primer for Clinicians" Journal of Osteopathic Medicine, vol. 118, no. 2, 2018, pp. 67-70. https://doi.org/10.7556/jaoa.2018.020
6. Kalant,H. "Medicinal Use of Cannabis: History and Current Status", Pain Research and Management, vol. 6, Article ID 469629, 12 pages, 2001. https://doi.org/10.1155/2001/469629

7. Solimini, R., Rotolo, M.C., Pichini, S., Pacifici, R. "Neurological Disorders in Medical Use of Cannabis: An Update". CNS & Neurological Disorders- Drug Targets 16, 527-533(7). 2017. https://doi.org/10.2174/1871527316666170413105421
8. Fiani, B., Sarhadi, K.J., Soula, M. et al. Current application of cannabidiol (CBD) in the management and treatment of neurological disorders. Neurol Sci 41, 3085–3098 (2020). https://doi.org/10.1007/s10072-020-04514-2
9. Waskow IE, Olsson JE, Salzman C, Katz MM. Psychological Effects of Tetrahydrocannabinol. Arch Gen Psychiatry. 1970;22(2):97–107. doi:10.1001/archpsyc.1970.01740260001001
10. Karniol I, G, Shirakawa I, Takahashi R, N, Knobel E, Musty R, E: Effects of Δ⁹-Tetrahydrocannabinol and Cannabinol in Man. Pharmacology 1975;13:502-512. doi: 10.1159/000136944
11. Pichini, S., Malaca, S., Gottardi, M., Pérez-Acevedo, A.P., Papaseit, E., Perez-Maña, C., Farré, M., Pacifici, R., Tagliabracci, A., Mannocchi, G., Busardò, F.P. UHPLC-MS/MS analysis of cannabidiol metabolites in serum and urine samples. Application to an individual treated with medical cannabis, Talanta, Volume 223, Part 2, 2021, 121772, ISSN 0039-9140, https://doi.org/10.1016/j.talanta.2020.121772.
12. Gray, R.A. and Whalley, B.J. (2020), The proposed mechanisms of action of CBD in epilepsy. Epileptic Disorders, 22: S10-S15. https://doi.org/10.1684/epd.2020.1135
13. Appendino, G., Gibbons, S., Giana, A., Pagani, A., Grassi, G., Stavri, M., Smith, E., Rahman, M.M. Antibacterial Cannabinoids from Cannabis sativa: A Structure−Activity Study. Journal of Natural Products 2008 71 (8), 1427-1430. DOI: 10.1021/np8002673
14. Blaskovich, M.A.T., Kavanagh, A.M., Elliott, A.G. et al. The antimicrobial potential of cannabidiol. Commun Biol 4, 7 (2021). https://doi.org/10.1038/s42003-020-01530-y
15. Gildea L, Ayariga JA, Ajayi OS, Xu J, Villafane R, Samuel-Foo M. Cannabis sativa CBD Extract Shows Promising Antibacterial Activity against Salmonella typhimurium and S. newington. Molecules. 2022 Apr 21;27(9):2669. doi: 10.3390/molecules27092669. PMID: 35566019; PMCID: PMC9099639.
16. Hong H, Sloan L, Saxena D, Scott DA. The Antimicrobial Properties of Cannabis and Cannabis-Derived Compounds and Relevance to CB2-Targeted Neurodegenerative Therapeutics. Biomedicines. 2022 Aug 12;10(8):1959. doi: 10.3390/biomedicines10081959.
17. Abichabki N, Zacharias LV, Moreira NC, Bellissimo-Rodrigues F, Moreira FL, Benzi JRL, Ogasawara TMC, Ferreira JC, Ribeiro CM, Pavan FR, Pereira LRL, Brancini GTP, Braga GÚL, Zuardi AW, Hallak JEC, Crippa JAS, Lanchote VL, Cantón R, Darini ALC, Andrade LN. Potential cannabidiol (CBD) repurposing as antibacterial and promising therapy of CBD plus polymyxin B (PB) against PB-resistant gram-negative bacilli. Sci Rep. 2022 Apr 19;12(1):6454. doi: 10.1038/s41598-022-10393-8.
18. Livermore, D.M. Has the era of untreatable infections arrived? Journal of Antimicrobial Chemotherapy, Volume 64, Issue suppl_1, September 2009, Pages i29–i36, https://doi.org/10.1093/jac/dkp255
19. WHO. (2020). Antimicrobial Resistance. https://www.who.int/healthtopics/antimicrobial-resistance
20. Ayariga, J.A., Gildea, L., Wu, H. and Villafane, R., 2021. The Ɛ34 Phage Tailspike Protein: An in vitro characterization, Structure Prediction, Potential Interaction with S. newington LPS and Cytotoxicity Assessment to Animal Cell Line. bioRxiv
21. Gildea, L., Ayariga, J.A., Robertson, B.K., Villafane R. P22 Phage Shows Promising Antibacterial Activity under Pathophysiological Conditions. Archives of Microbiology and Immunology 6 (2022): 81-100.

22. CDC. (2019). Antibiotic Resistance threats in the United States. https://www.cdc.gov/DrugResistance/Biggest-Threats.html
23. Slayton, R. B., Toth, D., Lee, B. Y., Tanner, W., Bartsch, S. M., Khader, K., Wong, K., Brown, K., McKinnell, J. A., Ray, W., Miller, L. G., Rubin, M., Kim, D. S., Adler, F., Cao, C., Avery, L., Stone, N. T., Kallen, A., Samore, M., Huang, S. S., Jernigan, J. A. (2015). Vital Signs: Estimated Effects of a Coordinated Approach for Action to Reduce Antibiotic-Resistant Infections in Health Care Facilities - United States. MMWR. Morbidity and mortality weekly report, 64(30), 826–831.
24. Kosgodage, U.S., Matewele, P., Awamaria, B. Kraev, I., Warde, P., Mastroianni, G., Nunn, A.V., Guy, G.W. (2019). Cannabidiol Is a Novel Modulator of Bacterial Membrane Vesicles. Frontiers in Cellular and Infection Microbiology, 9:2235-2988. https://doi.org/10.3389/fcimb.2019.00324
25. Martinenghi LD, Jønsson R, Lund T, Jenssen H. Isolation, Purification, and Antimicrobial Characterization of Cannabidiolic Acid and Cannabidiol from Cannabis sativa L. Biomolecules. 2020 Jun 12;10(6):900. doi: 10.3390/biom10060900.
26. Lukacik, P., Barnard, T. J., Keller, P. W., Chaturvedi, K. S., Seddiki, N., Fairman, J. W., Noinaj, N., Kirby, T. L., Henderson, J. P., Steven, A. C., Hinnebusch, B. J., & Buchanan, S. K. (2012). Structural engineering of a phage lysin that targets gram negative pathogens. Proc Natl Acad Sci U S A, 109(25), 9857-9862. https://doi.org/10.1073/pnas.1203472109
27. Lin, D. M., Koskella, B., & Lin, H. C. (2017). Phage therapy: An alternative to antibiotics in the age of multi-drug resistance. World J Gastrointest Pharmacol Ther, 8(3), 162-173. https://doi.org/10.4292/wjgpt.v8.i3.162
28. Ghosh, C., Sarkar, P., Issa, R., & Haldar, J. (2019). Alternatives to Conventional Antibiotics in the Era of Antimicrobial Resistance. Trends Microbiol, 27(4), 323-338. https://doi.org/10.1016/j.tim.2018.12.010
29. D'Accolti, M., Soffritti, I., Mazzacane, S., & Caselli, E. (2021). Bacteriophages as a Potential 360-Degree Pathogen Control Strategy. Microorganisms, 9(2). https://doi.org/10.3390/microorganisms9020261
30. Bryan, F.L.; Foodborne Diseases in the United States Associated with Meat and Poultry. J Food Prot 1 February 1980; 43 (2): 140–150. doi: https://doi.org/10.4315/0362-028X-43.2.140
31. Heinitz, M.L., Ruble, R.D., Wagner, D.E., Tatini, S.R; Incidence of Salmonella in Fish and Seafood. J Food Prot 1 May 2000; 63 (5): 579–592. doi: https://doi.org/10.4315/0362-028X-63.5.579
32. Park S. Y., C. L. Woodward, L. F. Kubena, D. J. Nisbet, S. G. Birkhold & S. C. Ricke (2008) Environmental Dissemination of Foodborne Salmonella in Preharvest Poultry Production: Reservoirs, Critical Factors, and Research Strategies, Critical Reviews in Environmental Science and Technology, 38:2, 73-111, DOI: 10.1080/10643380701598227
33. White, D.G., Zhao, S., Sudler, R., Ayers, S., Friedman, S., Chen, S., McDermott, P., McDermott, S., Wagner, D., Meng, J. (2001) The Isolation of Antibiotic-Resistant Salmonella from Retail Ground Meats. N Engl J Med 2001; 345:1147-1154. DOI: 10.1056/NEJMoa010315
34. Ardal C, B. M., Laxminarayan R, McAdams D, Outterson K et al. (2019). Antibiotic Development- Economic, regulatory, and societal challenges. National Review Microbiology.
35. Sharland MGS, H. B., Moja L, Pulcini C, Zeng M et al. (2019). Eml expert committee and antibiotic Working Group. Essential Medicines list becomes a global antibiotic stewardship tool. Lancet Infectious Disease. (19), 1278-1280.
36. Sustainable CBD LLC. (2020) Sustainable CBD LLC Standard of Procedures (SOP) 1-6.

37. Khunbustsri, D., Naimon, N., Satchasataporn, K., et al. (2022). Antibacterial Activity of Solanum torvum Leaf Extract with Oxacillin against MRSA Isolated from Dogs. Antibiotics, 11, 302. Doi: doi.org/10.3390/antibiotics11030302.
38. Ayariga, J.A.; Abugri, D.A.; Amrutha, B; Villafane, R. (2022). Capsaicin Potently Blocks *Salmonella typhimurium* Invasion of Vero Cells. Antibiotics, 11, 666. https://dio.org/10.3390.
39. Álvarez-Martínez, F.J., Barrajón-Catalán, E., Herranz-López, et al. (2021) Antibacterial plant compounds, extracts and essential oils: An updated review on their effects and putative mechanisms of action. Phytomedicine, Volume 90, 153626, ISSN 0944-7113, https://doi.org/10.1016/j.phymed.2021.153626.
40. Abichabki, N., Zacharias, L.V., Moreira, N.C. et al. Potential cannabidiol (CBD) repurposing as antibacterial and promising therapy of CBD plus polymyxin B (PB) against PB-resistant gram-negative bacilli. Sci Rep 12, 6454 (2022). https://doi.org/10.1038/s41598-022-10393-8.