

Cannabis sativa CBD Extract Shows Promising Antibacterial Activity against *S. typhimurium* and *S. newington*

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Abstract: Products derived from *Cannabis sativa* have gained increased interest and popularity. As these products become common amongst the public, the health and potential therapeutic values associated with hemp have become a premier focus of research. While the psychoactive and medicinal properties of Cannabis products have been extensively highlighted in literature, the antibacterial properties of CBD have not been explored in depth. This research serves to examine the antibacterial potential of CBD against *Salmonella newington* and *Salmonella typhimurium*. In this study we observed bacterial response to CBD exposure through biological assays, bacterial kinetics, and fluorescence microscopy. Additionally, comparative studies between CBD and ampicillin were conducted against *Salmonella typhimurium* and *Salmonella newington* to determine comparative efficacy. Furthermore, we observed potential resistance development of our *Salmonella* spp. against CBD treatment.

Keywords: *Salmonella*; Novel Antibacterial Agents; Cannabinoids

1. Introduction

Cannabis sativa, a member of the Cannabis genus and a species of the Cannabaceae family originated from Central Asia and is one of the oldest psychoactive plants known to man [1]. Since its discovery, *C. sativa* has been utilized recreationally, medicinally, and industrially for numerous applications. Early uses of *C. sativa* focused on its use as an industrial material to produce textiles, ropes, and paper products [2-4]. Throughout early history the use of *C. sativa* as an industrial product spread from Asia throughout Europe and Africa [5]. As industrial use of *C. sativa* became common, its use as a therapeutic against rheumatic pain, intestinal constipation, disorders of the female reproductive system, malaria, and other common health problems, can be traced back to ancient China where it was one of the oldest pharmaceuticals recorded [6]. Overtime the use of *C. sativa* as a therapeutic option was introduced across the western world and by the 19th century had gained attention from medical science. The first clinical conference on *C. sativa* in 1860 led to an increased interest in the research and development of medicinal products derived from *C. sativa* [2]. This interest in *C. sativa* drastically declined in 1942 when it was removed from the United States Pharmacopoeia and lost its status due to research suggesting a correlation between *C. sativa* and 'insanity' [7]. This decision eventually led to the United Nations designating Cannabis as an illegal psychotropic substance in 1971 at the Convention on Psychotropic Substances [8-9]. These designations and findings drastically reduced research progression on *Cannabis* and produced a negative stigma on the substance from the perspective of the public [10-11].

As we move towards the mid-21st century the acceptance of *Cannabis* and the potential of its byproducts has increased [12]. Continued research demonstrated the efficiency of *Cannabis* in the treatment of conditions including epilepsy, multiple sclerosis, Tourette's syndrome, and other neurological diseases [13-15]. Additionally, research has further characterized the active compounds, tetrahydrocannabinol (THC) and Cannabidiol (CBD), of *Cannabis* and their psychological and physiological effects on humans [16-19]. While clinical research has primarily focused on the efficacy of *Cannabis* against neurological disorders, a current gap in knowledge is the efficacy of *Cannabis* and its byproducts as antibacterial agents. The literature that is present does suggest that *Cannabis* and more specifically its active compound, CBD shows antibacterial function [20-21]. However, further research and characterization of this antibacterial function is crucial to the development of novel therapeutics against clinically relevant bacteria [22-23].

The increased prevalence and occurrence of antimicrobial resistance is a major health concern worldwide, making the development of alternative therapies a necessity [24-27]. The steady decrease in antibiotic efficacy alongside the decreased development of new antibiotics presents a major obstacle in the treatment of multidrug-resistant bacteria [28-29]. Antibiotic-resistant and multi-drug resistant bacterial infections account for roughly 2.8 million infections and 35,000 deaths annually in the United States alone [30]. Antibiotic-resistant bacterial infections, while a major threat to public health, also present a major economic burden to the United States resulting in an annual cost of 7.7 billion dollars [31]. As resistant bacterial species persist, the development of novel antibacterial treatment methods is essential to the preservation of public health. The purpose of this research is to determine if CBD extracted from *C. sativa* demonstrates antibacterial activity against a common gram-negative bacterial pathogen, *Salmonella*. CBD, initially not recognized as an active compound of Cannabinoids, has been shown to possess potential therapeutic benefits [32-34]. *Cannabis* is commonly associated with psychotic effects; we now know that these effects can be attributed to THC whereas CBD "exhibits no effects indicative of any abuse or dependence potential and to date, there is no evidence of public health related problems associated with the use of pure CBD" according to the World Health Organization [35]. Literature suggests that CBD's antibacterial function is carried out through the disruption of the cell membrane of both gram-positive and gram-negative bacteria [36]. The antibacterial activity of CBD against several bacterial pathogens including *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Clostridiosis difficile*, *Neisseria* spp., *Moraxella catarrhalis*, and *Legionella pneumophila* has been observed [20-21]. Study of CBD as an antibacterial agent requires further determination of the range of bacteria that CBD exhibits antibacterial activity against. Examining the potential of CBD against a clinically relevant pathogen such as *Salmonella* is essential to further expand our knowledge on CBD as a potential novel therapeutic.

Salmonella species are one of the most common and prevalent foodborne pathogens worldwide, found in several food products including poultry, seafood, and other fresh or processed meats [26-27, 37-39]. *Salmonella* accounts for 1.35 million infections, 26,500 hospitalizations, and 420 deaths annually in the United States alone [30]. *Salmonella* is a significant spoilage hazard, additionally, the increased prevalence of multi-drug resistant strains makes *Salmonella* a major threat to public health [25, 30]. The CDC reported in 2019 that *Salmonella typhimurium*, one of the species examined in this study, accounted for 59% of ampicillin-resistant *Salmonella* infections in the United States [30]. The continued use of broad-spectrum antibiotics could drastically increase the prevalence of antibiotic and multi-drug resistant *Salmonella* strains making the development of novel antibiotic alternatives a necessity [31, 40].

We have performed plate assays, fluorescence microscopy, and growth kinetic studies to determine the antibacterial activity of CBD extracted from *C. sativa* against *Salmonella typhimurium* and *Salmonella newington*. Additionally, we conducted comparative kinetic studies of the two *Salmonella* strains in the presence of CBD or a common broad-spectrum antibiotic, ampicillin. Finally, we examined resistance development of *S.*

typhimurium and *S. newington* against CBD treatment over an extended time. The results of these studies suggest that CBD does exhibit antibacterial activity against these *Salmonella* species further encouraging research and development of CBD as a potential anti-bacterial agent.

2. Results

2.1 Gas Chromatography

For confirmation of pure CBD in our *C. sativa* extract, Gas Chromatography (GC) was conducted. Figure 1 represents our sample (left) in comparison to the internal standard for CBD (right). In these readings we observe a strong single peak at the 8.23 min retention time (RT) in our sample. This is consistent with the expected RT of CBD at 8.22 min as shown in the internal control sample. The absence of other peaks represents the absence of other compounds or contaminants. Overall, we conclude through the GC analysis that our sample is pure CBD.

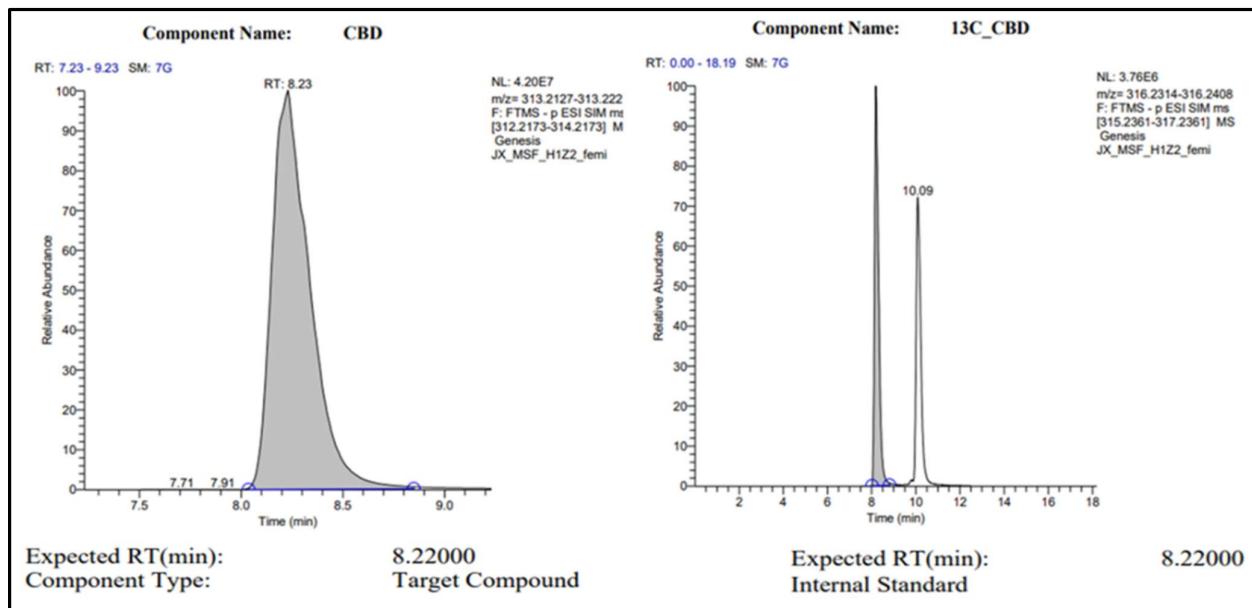


Figure 1. GC peaks confirms CBD as primary compound present within our *C. sativa* extract.

2.1 Plate Assays

To examine the potential antibacterial activity of our CBD against *S. typhimurium* and *S. newington* the Kirby-Bauer and spot assays were conducted. These assays allow us to visualize and quantify the inhibitory effects of CBD against our *Salmonella* strains. Both assays confirmed inhibitory activity of CBD against *S. typhimurium* and *S. newington*. In the Kirby-Bauer assay we observed zones of inhibition (ZOI) around the CBD treated disks suggesting inhibition of bacterial growth due to exposure to CBD (Fig.2C). These results also suggest a dose-dependent inhibition due to CBD treatment with ZOIs decreasing as CBD concentration decreased (Fig.2A, Fig.2B). The results of the spot assay further confirmed the dose-dependent nature of CBD's inhibitory activity. As CBD

concentration decreased, we see an increase in the density of bacterial colonies in both *S. typhimurium* and *S. newington* (Fig.2D). This data also suggested that *S. newington* was more susceptible to CBD treatment than *S. typhimurium* due to the larger ZOIs (Fig.2B) and lower colony density (Fig.2D). 132
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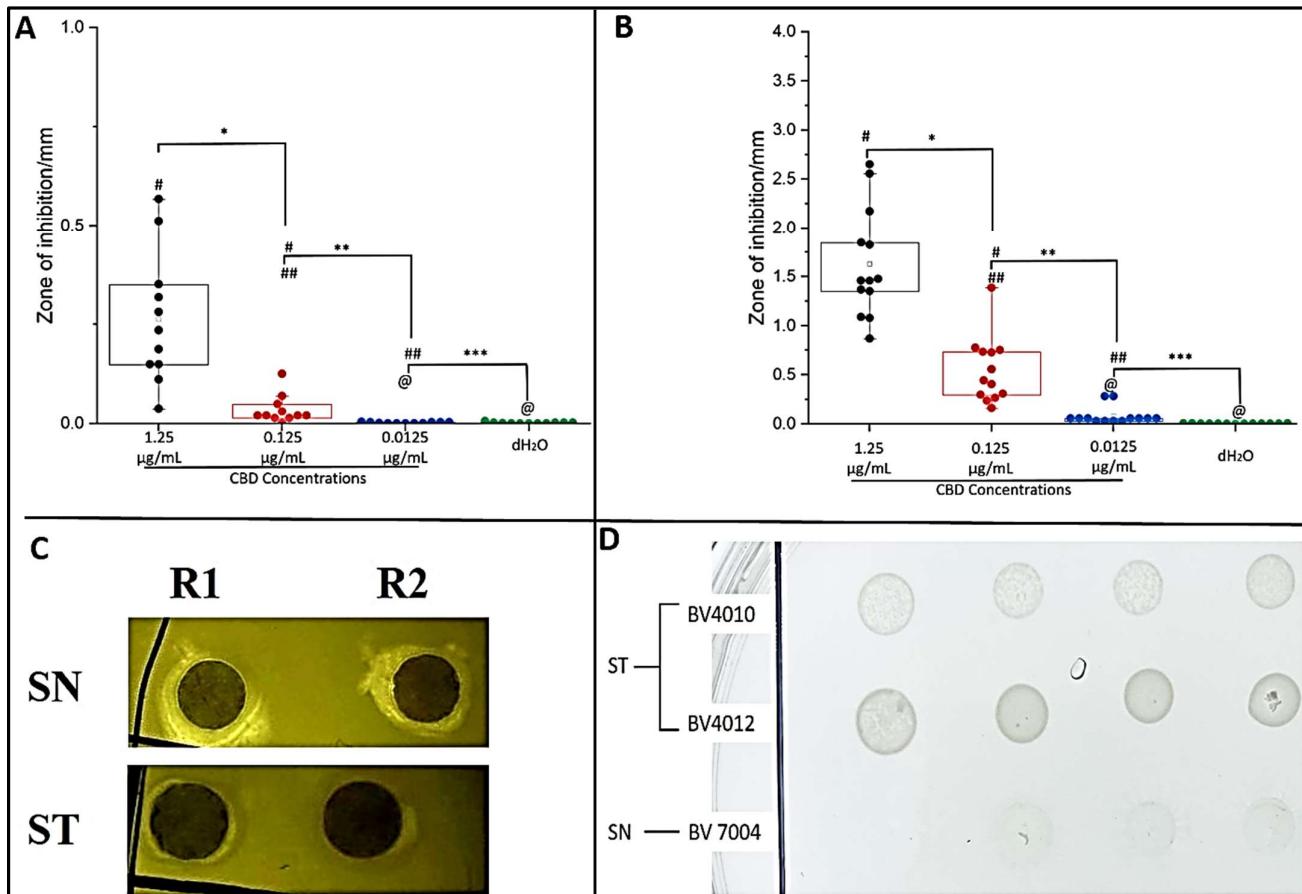


Figure 2. Plate Assays confirm inhibitory activity of CBD. Quantitative analysis of Kirby-Bauer assay results of *S. typhimurium* (Fig.2A) and *S. newington* (Fig.2B). Images from Kirby-Bauer assay and CBD produced ZOIs against *S. typhimurium* and *S. newington* (Fig.2C). Images from spot assay featuring *S. typhimurium* and *S. newington* treated with decreasing concentrations (left to right) of CBD (Fig.2D). 137
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2.2 CBD Extract Reduces Bacterial Growth of *Salmonella typhimurium* and *Salmonella newington* 143 144

To determine the effect of CBD on *S. typhimurium* and *S. newington*, cultures were treated with CBD at concentrations of 1.25 $\mu\text{g/mL}$, 0.125 $\mu\text{g/mL}$, 0.0125 $\mu\text{g/mL}$, or 0.00125 $\mu\text{g/mL}$. The optical density (OD600) was recorded using a spectrometer (Molecular Devices SpectraMax® ABS Plus) hourly for 6 h. Both *S. typhimurium* and *S. newington* cultures treated with CBD showed a significant reduction in OD600 within 6 h of treatment (Fig.3A, Fig.3B). It was even observed that in *S. newington* CBD, at concentration as low as 0.0125 $\mu\text{g/mL}$, reduced bacterial growth over the 6-h period. These results suggest that *S. newington* might have a greater susceptibility to CBD than *S. typhimurium* whose MIC was 145
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0.125 $\mu\text{g/mL}$. The reduction of OD600 signifies that CBD extract does exhibit antibacterial characteristics. The antibacterial effect on *S. typhimurium* and *S. newington* was observed to be dose-dependent with the OD600 increasing in correlation with decreased concentrations of CBD. 153
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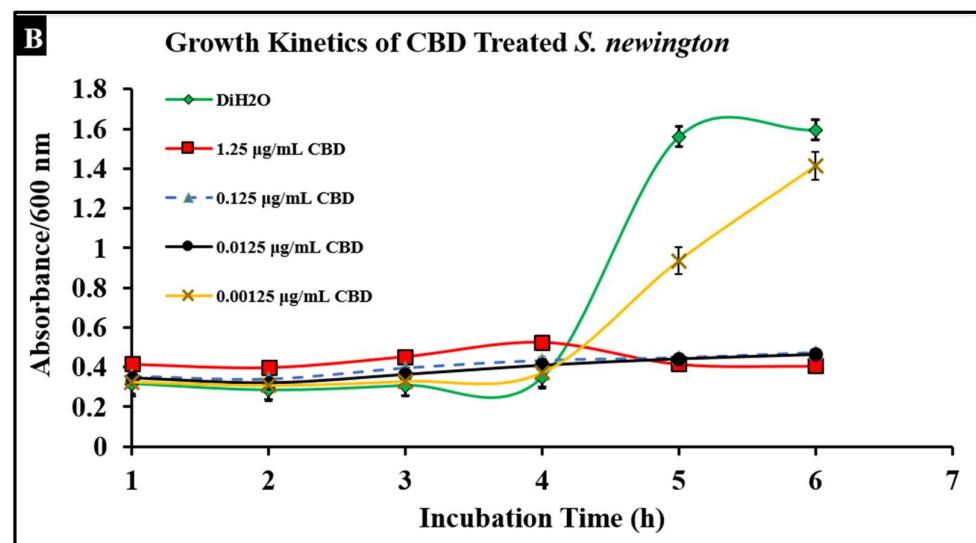
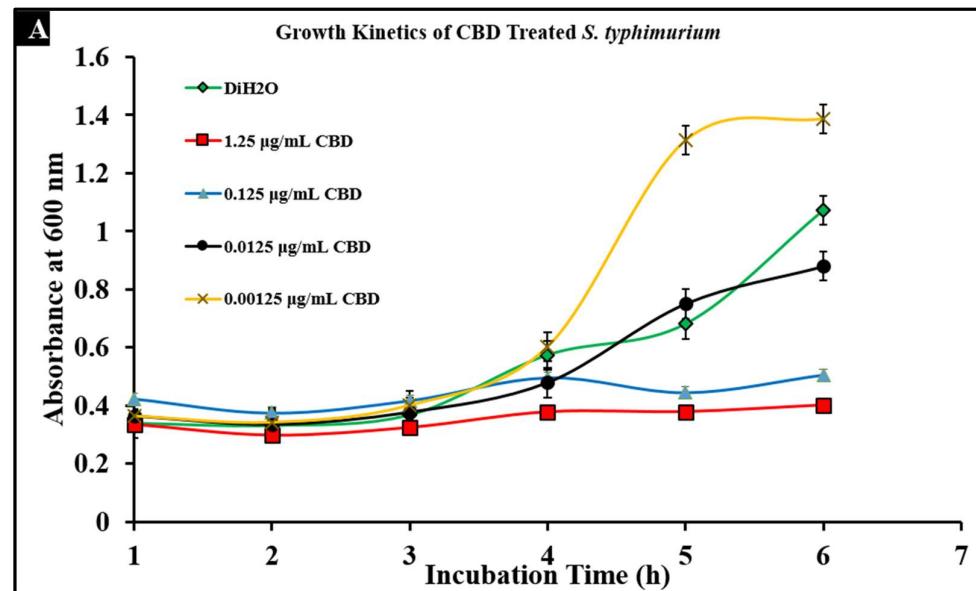


Figure 3. Treatment of lag-phase *S. typhimurium* (Fig.3A) and *S. newington* (Fig.3B) with CBD. The effect of several CBD dilutions on OD600 was recorded in triplicate. 159
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2.3 Fluorescent DAPI staining of CBD Treated *Salmonella* Cells

To examine the effect of CBD treatment on *Salmonella* cells, *S. typhimurium* and *S. newington* samples were treated with CBD at concentrations of 1.25 $\mu\text{g/mL}$, 0.125 $\mu\text{g/mL}$, or 0.0125 $\mu\text{g/mL}$. These samples were visualized using immunofluorescent 4',6-diamidino-2-phenylindole (DAPI) staining. This staining technique emits fluorescence when the stain binds to A-T rich DNA. This stain is membrane impermeable thus, 163
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fluorescence represents compromised membrane integrity. DAPI staining confirmed that 168
CBD treatment resulted in degradation of membrane integrity after 5 min and 30 min of 169
treatment (Fig.4A, Fig.4B). Quantitative analysis using densitometry of these images 170
showed increased DAPI fluorescence from the 5 min to the 30 min time points (Fig.4C). 171
These images all indicate that CBD can successfully damage *Salmonella* cells outer 172
lipopolysaccharide membranes. 173

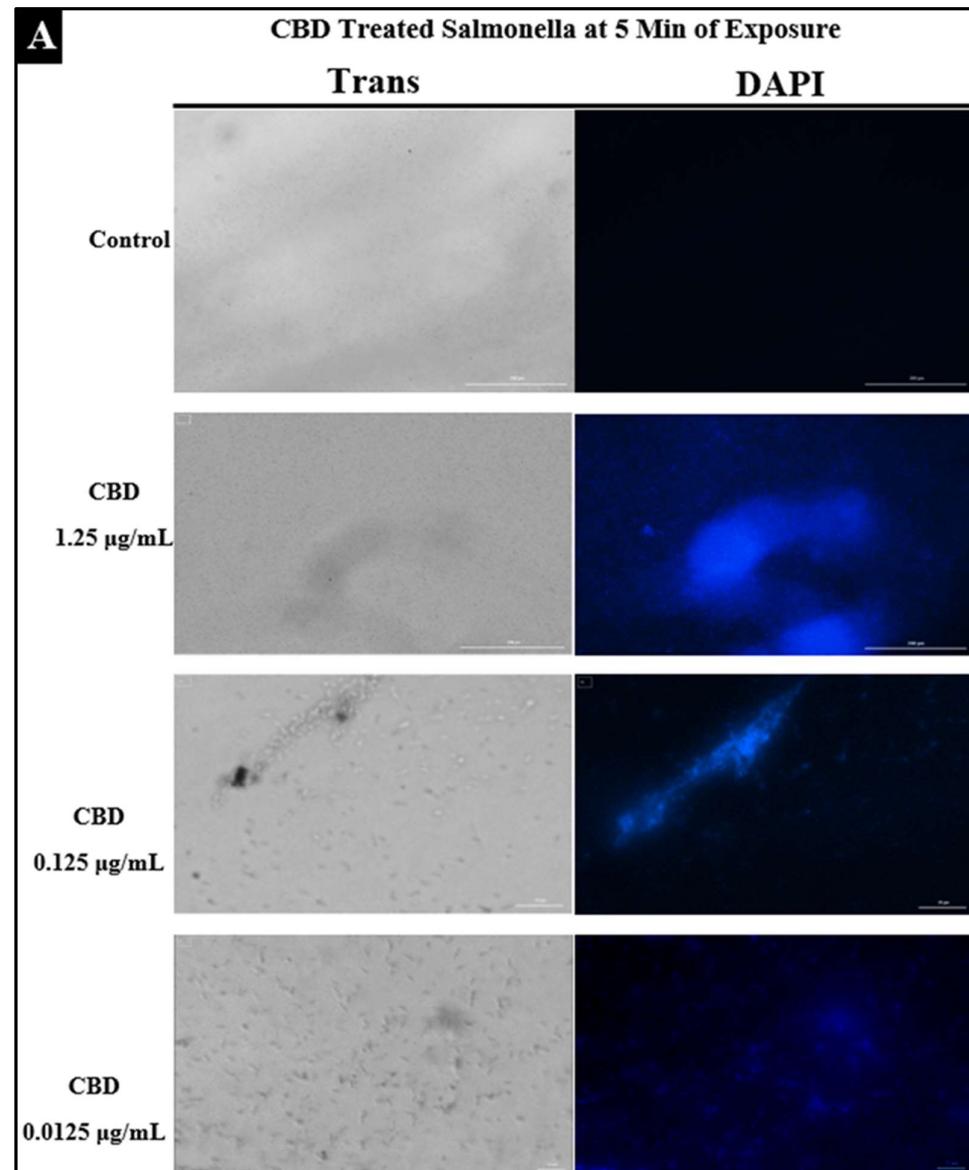


Figure 4A. Fluorescence microscopy of CBD treated *Salmonella* cells stained with DAPI. 175
Images were acquired at the time points of 5 min. DAPI staining was utilized to assess 176
membrane integrity following treatment with CBD. 177

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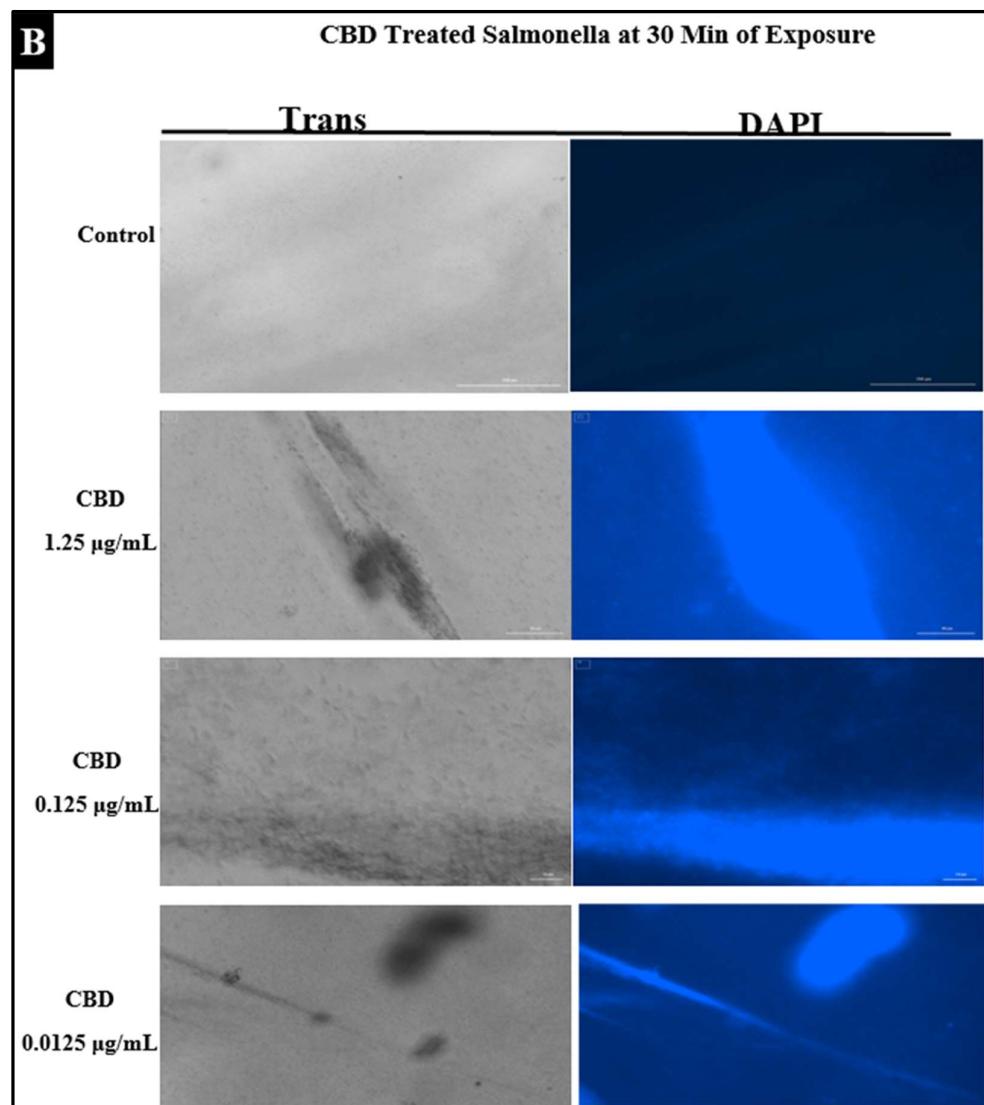


Figure 4B. Fluorescence microscopy of CBD treated *Salmonella* cells stained with DAPI. Images were acquired at the time points of 30 min. DAPI staining was utilized to assess membrane integrity following treatment with CBD.

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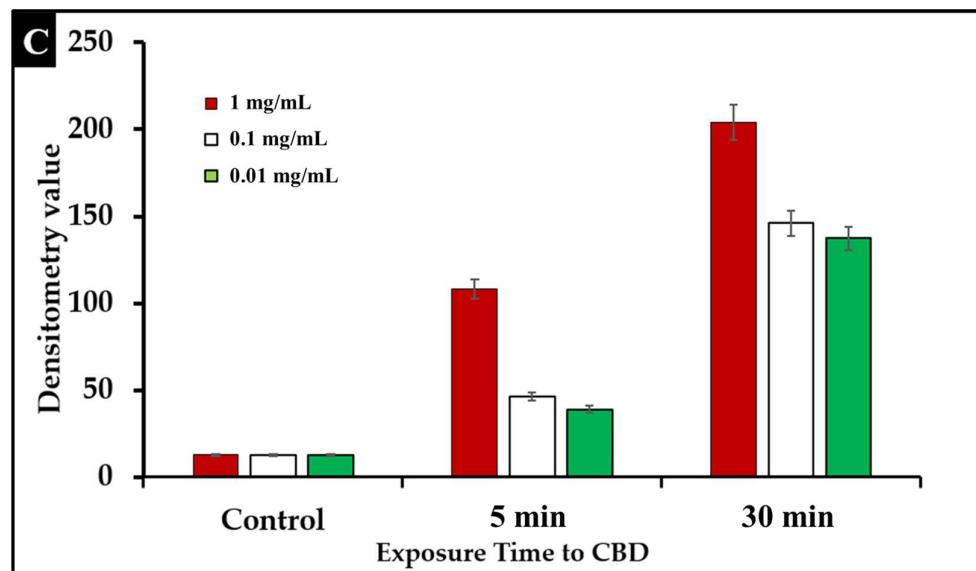
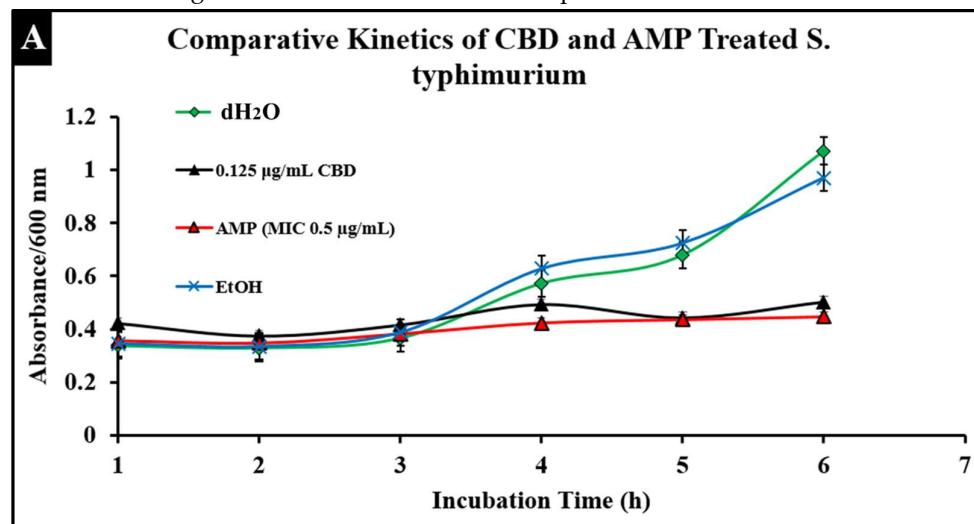


Figure 4C. Quantitative analysis of membrane integrity via densitometry analysis of fluorescence after DAPI staining.

2.4 Comparative Study of CBD and Antibiotic Treatment Against *Salmonella*

Salmonella infections are typically treated with broad spectrum antibiotics such as ampicillin, however the development of resistance to these treatments has become more prevalent thus, increasing the need for alternative treatments. To compare conventional antibiotics to CBD we conducted comparative kinetic studies to observe antibacterial activity. In this study *Salmonella* strains *S. typhimurium* and *S. newington* at an OD600 of 0.5 were treated with the minimum inhibitory concentration (MIC) of ampicillin (0.5 μ g/mL) or CBD (0.125 μ g/mL). Results suggested that CBD and ampicillin both successfully inhibited *Salmonella* growth in both strains (Fig.5). In comparison both treatments resulted in a similar OD600 after 6 h of treatment suggesting CBD was able to inhibit bacterial growth to an extent similar to ampicillin.



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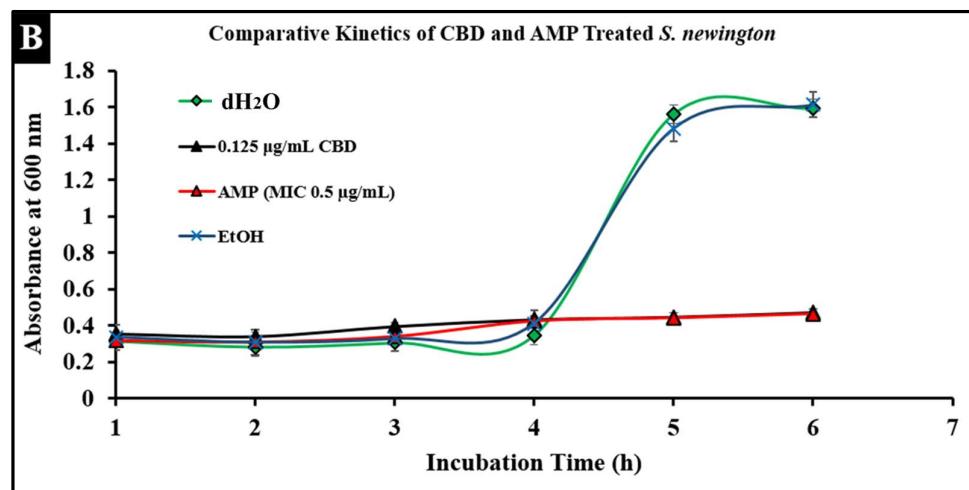


Figure 5. Comparative efficacy of CBD and antibiotic treatment of *Salmonella typhimurium* (Fig.5A) and *Salmonella newington* (Fig.5B). *Salmonella* cells were cultured overnight and then treated with either CBD or ampicillin. OD₆₀₀ was measured hourly over a 6-h time period to observe bacterial growth following treatment.

2.5 Developed Resistance of *Salmonella* to CBD Treatment

A major concern with conventional antibiotic treatments is the development of resistance. To observe the development of resistance in *Salmonella* against CBD treatment extended kinetic studies were conducted over a span of 48 h. *Salmonella* strains *S. typhimurium* and *S. newington* were treated with ampicillin (MIC 0.5 µg/mL) or CBD (1.25 µg/mL, 0.125 µg/mL, 0.0125 µg/mL, or 0.00125 µg/mL). Results confirmed a developed resistance to CBD in both *Salmonella* species (Fig.6). At concentrations of 1.25 µg/mL and 0.125 µg/mL of CBD resistance development was not observed until the 48 h time point. These results suggest that CBD was effective in killing *Salmonella* and decreasing the rate of resistance development in both *Salmonella* strains over the initial 24 h time period. At 0.0125 µg/mL, 0.00125 µg/mL, and 0.000125 µg/mL concentrations of CBD there was no significant reduction in bacterial growth and resistance was developed after only 12 h of exposure in both *Salmonella* strains. These results suggest that both *Salmonella* strains were able to develop resistance quickly to low concentrations of CBD.

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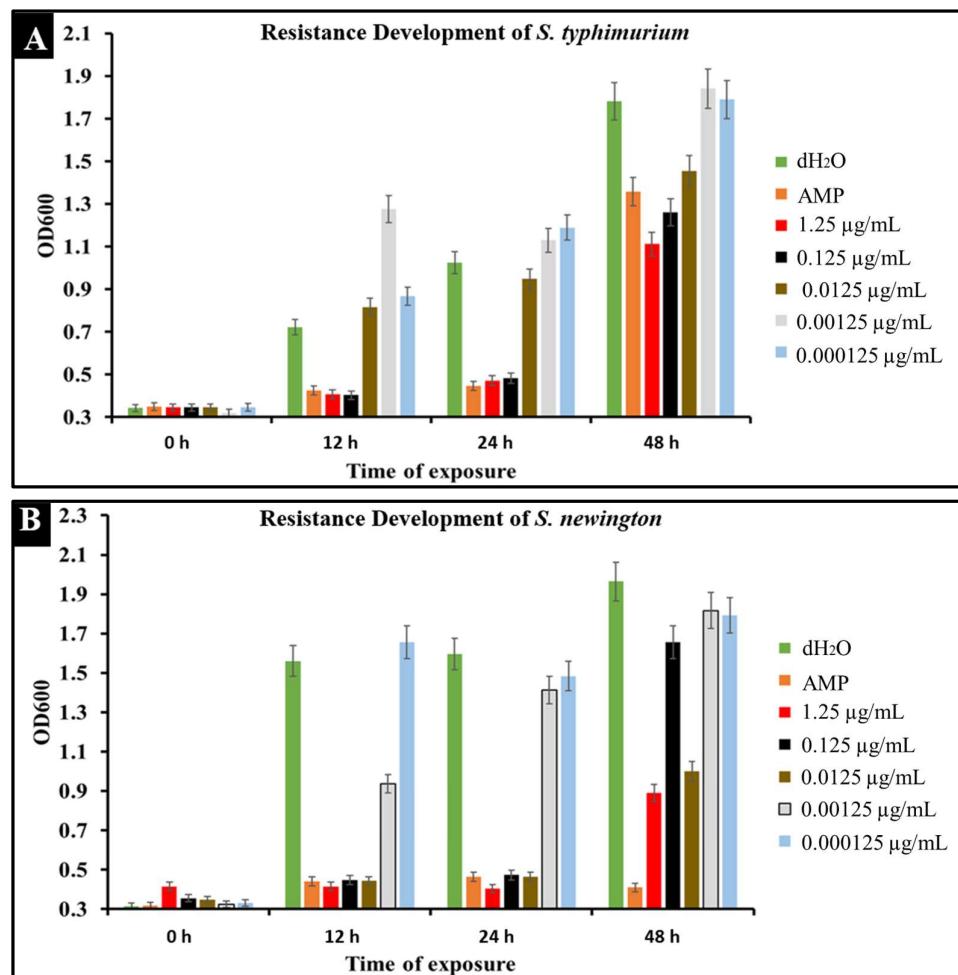


Figure 6. Development of resistance to CBD and ampicillin treatment by *S. typhimurium* (Fig.6A) and *S. newington* (Fig.6B) examined through extend kinetics. Bacterial cultures were treated with either ampicillin, CBD dilutions, or dH₂O. OD₆₀₀ was recorded over a period of 48 h.

1. Discussion

The rapid increase of antibiotic and multidrug resistant bacterium over the early 21st century is a major threat to public health [30]. Knowing this, the study of potentially efficacious alternative therapeutics has been a topic of growing interest. *C. sativa* products, while typically associated with the treatment of neurological disorders, have shown promise as antibacterial agents against several notable pathogens [20-21]. Of the multiple metabolites of *C. sativa*, research suggests that CBD is the most promising [32-34]. This compound, unlike THC, holds no psychoactive properties but does possess antioxidant, anti-inflammatory, and antibacterial properties [22, 41, 42]. CBD's antibacterial potential has shown some promise; however, research is relatively limited. Further investigation and confirmation of the specificity of this antibacterial activity is crucial as the field of medicine searches for new viable therapeutics for resistant bacterial infections.

C. sativa products have exhibited a wide variety of applications in numerous fields. This study helps expand our knowledge of *C. sativa* derived CBD as an antibacterial agent against two common gram-negative pathogenic *Salmonella* strains. *Salmonella* spp. have significance in terms of resistance to antibiotics and standard treatment protocols making these infections a major threat to the preservation of public health [25, 28, 30]. Discovery

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and development of novel antibacterial agents such as CBD are a major step towards the future of therapeutics in a world where antibiotics are no longer efficacious and cost effective [20, 21]. This study serves primarily to determine the antibacterial potential of CBD against *S. typhimurium* and *S. newington* through plate assays, fluorescence microscopy, and kinetic assays. Additionally, this study examines the comparative antibacterial activity of CBD and ampicillin as well as resistance development of *S. typhimurium* and *S. newington* against CBD treatments.

In this study, we confirm that CBD does exhibit antibacterial activity against *S. typhimurium* and *S. newington*. This inference was derived from a combination of plate assays, fluorescence microscopy, and bacterial growth kinetic assays and thus allowed us to propose a potential mechanism of this antibacterial activity. Plate assays consisted of the Kirby-Bauer assay and the Spot assay, both of which served as initial confirmation of bacterial inhibition of *S. typhimurium* and *S. newington*. Once this inhibitory activity was observed, a fluorescence microscopy assay for membrane integrity was utilized. This assay utilized DAPI stain, a membrane impermeable DNA binding stain. Fluorescence of the DAPI stain confirmed that CBD treatment of *S. typhimurium* and *S. newington* resulted in a loss of membrane integrity and cell death. Thus proofing our hypothesis that the CBD treatment leads to membrane disruption, and this mechanism has been reported by other researchers as the antibacterial mechanism of action of CBD [36]. Finally, kinetic assays were conducted to examine the effect of CBD treatment on lag-phase *S. typhimurium* and *S. newington* over a span of 6 h. Once again, it was observed that CBD was effective in growth inhibition of both species in 6 h period with a MIC of 0.0125 μ g/mL in *S. typhimurium* and 0.125 μ g/mL in *S. newington*. These findings confirm that CBD does possess antibacterial activity through mechanisms similarly described in other gram-negative bacterial species [20 -21, 36].

An important facet of this study was the comparison between CBD and broad-spectrum antibiotic treatment. In this study we examined the comparative kinetics between CBD treatment and ampicillin treatment of *S. typhimurium* and *S. newington*. Results of these kinetic studies concluded that both CBD and ampicillin at their MIC concentrations are successful at inhibition of *S. typhimurium* and *S. newington* growth. It is important to note that the MIC concentration of ampicillin (0.5 μ g/mL) is significantly higher than the MIC of CBD (0.125 μ g/mL) (Fig. 5). To further investigate the comparative efficacy of a novel therapeutic agent (i.e. CBD in this study) and a standard treatment (such as ampicillin), it was important to examine the resistance development of the pathogen to the therapeutic agents. To examine the resistance of *S. typhimurium* and *S. newington* against CBD and ampicillin treatments, extended growth kinetics under different dosages of CBD were carried out over a 48 h period (see Fig. 6). These results varied across the two strains of *Salmonella* that were used. In *S. typhimurium*, CBD concentrations of 1.25 μ g/mL and 0.125 μ g/mL, were able to inhibit bacterial growth over the span of 24 h after a single treatment. These results are similar to the growth kinetics we observed with ampicillin treatment after 24 h. Whereas lower concentrations of CBD showed a rapid rise in growth following 12 h of treatment, suggesting a rapid development of resistance. After 48 h of exposure to CBD treatment *S. typhimurium* had developed resistance, suggested by rapid rise in OD600 at the 48 h time point, to all CBD treatments and the ampicillin treatment. This result is consistent with literature on *S. typhimurium*, which accounts for 59% of all ampicillin-resistant *Salmonella* infections in the United States [30]. These results raise the question of what mechanism does *S. typhimurium* develop to resist the antibacterial activity of CBD. Literature has suggested several mechanisms for *S. typhimurium* resistance to antibiotics including the multidrug efflux pump AcrAB, OXA-1 β -lactamase, and other beta-lactamase genes (*bla*). Ampicillin, a beta-lactam class antibiotic, kills bacteria through binding to penicillin-binding-proteins in the cytoplasmic membrane [43-45]. This mechanism of resistance relies on the inner mechanisms of the bacterial cell, whereas we hypothesize that CBDs antibacterial activity is a result of membrane disruption. In this work, we demonstrated that the antibacterial activity of CBD was generated through membrane

integrity disruption. Therefore, we suggest that *S. typhimurium* resistance to CBD might be conferred through a resistance mechanism different from that of ampicillin-resistance mechanism of this bacteria. This could be a significant in the application of CBD as a therapeutic agent against ampicillin resistant *S. typhimurium* or in a potential co-therapy designed with CBD and ampicillin. Co-therapies typically reduce the ability of bacteria to persist due to bacteria having to rapidly develop multiple resistance mechanisms [27, 46-49]. This further buttress the need to employ CBD in a potential CBD-antibiotic co-therapy once the resistance mechanism of CBD is fully understood. The extended kinetics of *S. newington* revealed that this strain was more susceptible to CBD treatments of 1.25 µg/mL, 0.125 µg/mL, and 0.0125 µg/mL 24 h after treatment. However, at the 48-h time point there was a strong development of resistance in all CBD treatments but no resistance development to ampicillin. These results suggest that the mechanism of resistance is different between ampicillin and CBD (Fig. 6). Further study to characterize the resistance developed by *S. typhimurium* and *S. newington* against CBD is important to understand how to responsibly develop this potential therapeutic agent.

Previous literature has outlined the potential of CBD against several, mostly gram-positive bacterial pathogens including *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Clostridiosis difficile* [20-21]. This study focused on a relevant gram-negative pathogen, *Salmonella*, whose tolerance of CBD has not previously been studied. As we continue to explore potential antibacterial agents it is essential that we explore the effectiveness of these agents against a multitude of diverse bacterial pathogens. Considering this, our study confirms that CBD does have antibacterial activity against two gram-negative *Salmonella* strains, filling a valuable gap in our knowledge of CBD as an antibacterial agent. While this study illuminates the potential of CBD as a therapeutic and fills a void in current literature, future work is necessary for further development of this bioactive compound as a therapeutic agent.

While our study was successful in determining the presence of CBD's antibacterial activity against *S. typhimurium* and *S. newington* there were limitations. One limitation is the specific resistance mechanism of *S. typhimurium* and *S. newington* against CBD was not investigated in this study. This study simply determined the ability of our *Salmonella* strains to develop resistance against the CBD treatments administered and did not to define the mechanism of resistance. The development of CBD as a novel therapeutic option will require further study and characterization. Some relevant studies for the progression of CBD as a potential therapeutic include resistance mechanisms to CBD, cytotoxicity of CBD especially to in co-therapy situations, immunological response to CBD treatment, CBD function in pathophysiological conditions, and *in vivo* models. These future studies will serve to further expand our knowledge of CBD as an antibacterial agent with potential therapeutic benefit.

4.0 Materials and Methods

4.1 CBD Extraction

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 200-500 microns and underwent CO₂ extraction in an Apeks Transformer for subcritical extraction (Gibraltar Industries Inc., Buffalo, NY, USA). Subcritical extraction was carried out at a target pressure of 1,200 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 has been carried out Apeks Transformer was utilized for supercritical extraction (Gibraltar Industries Inc., Buffalo, NY, USA). Supercritical extraction was carried out at a target pressure of 1,800 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 359
an extraction time of approximately 1-2 h per pound of material. The resulting material 360
then underwent winterization through addition of ethanol to the crude extract. This sam- 361
ple was frozen and then filtered through Buchner funnels and remaining ethanol is evap- 362
orated using a Heidolph rotary evaporator (Heidolph Instruments GmbH & Co.KG, Kel- 363
heim, Germany). Distillation was carried out using the Lab Society 5L short path distilla- 364
tion unit (Lab Society®, Boulder, CO, USA) for further refinement. The resulting product 365
of this procedure is winterized cannabinoid [50]. 366

4.2 Gas Chromatography Analysis of CBD 367

CBD extracts were analyzed by Gas Chromatograph (GC)/ Mass Spectrometry (MS) 368
using Agilent 6890N GC and Agilent 5975 MS (Agilent Technologies Inc., Santa Clara, CA, 369
USA) with a Restek Rxi-5Sil MS with integra guard column (15 m, 0.250 mmID, 0.25 µm 370
df) (Restek Corporation, Centre County, PA, USA). A solution of Restek Qualitative Re- 371
tention Time Index Standard (Restek Corporation, Centre County, PA, USA) was used to 372
create the retention time index. The temperature of the injection port was set at 280 °C and 373
the helium gas flow was constant at 1.1 mL/min. The samples were injected in split mode 374
(2:1) with a volume of 1µL of sample. The GC oven temperature was programmed as fol- 375
lows: initial temperature of 70 °C for 4 min, ramp to 200 °C at 20 °C/min, ramp to 300 °C 376
at 8 °C/min, ramp to 325 °C at 50 °C/min with a 5-minute hold, thus requiring a total run 377
time of 30.5 min. The MS transfer line was set to 250 °C, source to 230 °C, and quads to 378
150 °C. The raw data was processed and analyzed using Agilent Enhanced ChemStation 379
software (Agilent Technologies Inc., Santa Clara, CA, USA). The NIST 2.0 library was used 380
with AMDIS for compound identification. 381

4.3 Plate Assays for Antibacterial Screening of CBD 382

To qualitatively determine the antibacterial potential of CBD against *Salmonella typhi- 383
murium* and *Salmonella newington* plate assays were utilized. Prior to the assays, CBD was 384
serially diluted to the concentrations of 1.25 µg/mL, 0.125 µg/mL, 0.0125 µg/mL, or 0.00125 385
µg/mL. Bacterial strains *S. typhimurium* and *S. newington* were incubated until the late log- 386
phase (OD>1) in sterile Luria broth (BD Difco, Franklin Lakes, NJ, USA). 387

To conduct the Kirby-Bauer assay plates were inoculated with overnight cultures of 388
either *S. typhimurium* or *S. newington* and top agar (BD Difco, Franklin Lakes, NJ, USA) to 389
create a bacterial lawn. These plates are then divided into quarters representing the four 390
CBD dilutions (1.25 µg/mL, 0.125 µg/mL, 0.0125 µg/mL, or 0.00125 µg/mL). Sterile paper 391
discs were soaked in a designated dilution of CBD or treated with dH₂O (Control) and 392
then applied to the agar plates in triplicate. The result is three discs per CBD dilution on 393
the agar plate. Plates were then incubated at 37°C for 24 h, and bacterial growth was ob- 394
served to determine zones of inhibition (ZOI) around the CBD treated discs [51-53]. ZOIs 395
were quantitatively assessed using ImageJ (NIH Image, Bethesda, Maryland). This assay 396
was completed in triplicate. 397

For further confirmation of antibacterial activity, spot assays were conducted using 400
lag-phase *S. typhimurium* and *S. newington* cultures (OD>1). Sterile agar plates were inoc- 401
ulated with a four 10µL aliquots of *S. typhimurium* or *S. newington*. Each 'dot' was then 402
inoculated with 10µL of one of the four dilutions (1.25 µg/mL, 0.125 µg/mL, 0.0125 µg/mL, 403
or 0.00125 µg/mL) of CBD. Each culture was also spotted and treated with 10uL of dH₂O 404
as the control. The plate was then incubated for 24 h at 37°C and observed for inhibition 405
of bacterial growth [54]. This assay was completed in triplicate. 406

4.4 Fluorescence Microscopy of CBD Treated *Salmonella* Cells 407

To visualize the effects of CBD treatment on *Salmonella* cells fluorescence microscopy 408
was utilized. *Salmonella* cells were fixed and stained using DAPI (4',6-diamidino-2-phe- 409
nylindole) to determine cell membrane integrity. DAPI stain binds exclusively to dsDNA 410
which is only accessible as a result of compromised membrane integrity and expulsion of 411
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the cytoplasmic material including the nucleus out of the cell. Visualization of DAPI fluorescence under the fluorescence microscope (Biotek Cytation™ 3 Automated Fluorescence Microscope) (Agilent Technologies Inc., Santa Clara, CA, USA) confirms cell membrane damage and hence cell death. *Salmonella* cells were treated with CBD dilutions (1.25 μ g/mL, 0.125 μ g/mL, or 0.0125 μ g/mL) or left untreated (control). Following treatment cells were fixed at either 5 min or 30 min and assessed using fluorescence microscopy.

4.5 Bacterial Growth Kinetics

To study bacterial growth kinetics a 96 well plate (Fisherbrand™, Fisher Scientific, Fair Lawn, NJ, USA) was inoculated with 180 μ L of overnight bacterial cultures of either *S. typhimurium* or *S. newington* at an OD600 \approx 0.5. The cultures were then incubated at 37°C with rotary shaking at 121 rpm. Measurements of bacterial density (OD600) were taken every hour for 6 h and again at the 12 h, 24 h, and 48 h time points using a spectrometer (Molecular Devices SpectraMax® ABS Plus) (Molecular Devices LLC., San Jose, CA, USA). This experiment was completed in triplicate.

4.6 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 either *S. typhimurium* or *S. newington* at an OD600 \approx 0.5. These cultures were then treated with varying concentrations of CBD (1.25 μ g/mL, 0.125 μ g/mL, 0.0125 μ g/mL, or 0.00125 μ g/mL). The cultures were then incubated at 37°C with rotary shaking at 121 rpm. Measurements of bacterial density (OD600) were taken hourly for 6 h and at the 12 h, 24 h, and 48 h time points using a spectrometer (Molecular Devices SpectraMax® ABS Plus). This experiment was completed in triplicate.

4.7 Comparative Bacterial Growth Kinetics in the Presence of Ampicillin or CBD

To study how Ampicillin affects bacterial growth kinetics three 96 well plates were inoculated with overnight bacterial cultures of either *S. typhimurium* or *S. newington* at an OD600 \approx 0.5. These cultures were then treated with either Ampicillin (0.5 μ g/mL) or CBD (0.125 μ g/mL). The cultures were then incubated at 37°C with rotary shaking at 121 rpm. Measurements of bacterial density (OD600) were taken hourly for 6 h and at 24 h and 48 h time points using a spectrometer (Molecular Devices SpectraMax® ABS Plus). This experiment was completed in triplicate.

4.8 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 performed using OriginPro Plus version 2021b (OriginLab Corporation, North Hampton, MA, USA).

5. Conclusion

As this facultative anaerobe continues to cause serious public health problems, the need to investigate *Salmonella* has received much scientific scrutiny [55-58]. In this study, we demonstrated the antibacterial activity of CBD against two relevant pathogenic bacteria, *S. typhimurium* and *S. newington*. Despite the scarce knowledge of the molecular mechanisms of CBD's mode of action, we propose that the antibacterial activity might be due to membrane integrity disruption, and this was verified through the utilization of plate assays, fluorescence microscopy, and kinetic studies. These experiments confirmed that CBD has antibacterial activity against our target bacteria. Additionally, our comparative studies showed that CBD has antibacterial activity similar to ampicillin with a MIC

roughly one-fifth of ampicillins. Finally, we observed the resistance development of *S. typhimurium* and *S. newington* against CBD treatment. Resistance development was observed after 48 h but results suggest that *Salmonella* resistance to CBD is conferred through a different mechanism than antibiotic resistance. These results pose the question of CBD-antibiotic co-therapy as a potential novel application. This study further progresses our current knowledge on the effectiveness of CBD as an antibacterial agent and demonstrates the effectiveness of CBD against gram-negative bacterium, *S. typhimurium* and *S. newington*. 464
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