THE EFFECT OF CURCUMIN ON FRANCISELLA AND FTSZ

by

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Fairfax, VA
The Effect of Curcumin on Francisella and FtsZ

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at George Mason University

by

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I would like to thank my advisor, Dr. Monique van Hoek, for mentoring, advising and helping perfect various thesis drafts. I would also like to thank my committee, Dr. Barney Bishop and Dr. Iosif Vaisman. Additionally, I would like to thank the entire van Hoek lab who also helped me complete this project and George Mason University for the teaching assistantships that supported me through this degree. Also, thanks to my parents and partner for their continuous support.
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ABSTRACT

THE EFFECT OF CURCUMIN ON FRANCISELLA AND FTSZ

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This thesis assesses the effect of curcumin on *Francisella (F.) novicida* and the protein FtsZ. Curcumin is a polyphenolic compound found in turmeric that is widely used as a spice and an ayurvedic medicine in South-East Asia. *F. novicida* is the non-virulent strain of *F. tularensis* and is commonly used as a model organism as it can be used in a BSL2 laboratory. Antibacterial assays demonstrated that curcumin inhibited bacterial growth at 16 µg/ml (Minimal Inhibitory Concentration) and did not affect biofilm formation in *F. novicida*. Curcumin showed a dose-dependent relationship in significantly reducing bacterial load in *F. novicida*-infected mouse macrophages, suggesting that curcumin is able to enter the macrophages and inhibit bacterial growth. – Curcumin showed synergy with three antibiotics doxycycline, erythromycin and ciprofloxacin, suggesting that a combined approach to therapy might be advantageous in treating tularemia. Because of the role of FtsZ in regulating cell division through its interaction with minC, minD, and minE, we examined whether curcumin treatment may alter *Francisella* morphology, or
cause filamentation, as is reported for Bacillus. Curcumin treatment did not show significant changes in bacterial morphology or size but showed an increase in granularity as measured by Fluorescent Activated Cell Sorting. Bioinformatics analysis of curcumin and FtsZ interaction was modeled for the F. novicida protein, and showed a similar docking site with GTP as reported for E. coli FtsZ. A consensus of residues 138 and 142 as being involved in GTP binding was observed using multiple software confirming that curcumin and GTP bind at the same site in FtsZ. Targeting FtsZ with Curcumin provide novel potential target to treat this bacterial infection; however, low bioavailability of curcumin poses a challenge in using curcumin as a therapeutic.
CHAPTER ONE: INTRODUCTION

*Francisella*

Tularemia is a plague-like disease colloquially known as rabbit fever. The bacteria causes Tularemia and was isolated in 1911. It is a small gram-negative bacterium named *Bacterium tularensis*. Natural infections have been reported in vertebrates and the disease occurs only in the northern hemisphere[3]. The number of cases of tularemia have declined during the twentieth century in the United States, from several thousands in 1930s to a few hundred in 1980s. However, the figures may be underrepresented due to difficulties in diagnosing and the benign nature of disease caused by some strains. *Francisella tularensis* causes Tularemia in wild animals and humans[3]. Arthropod vectors transmit *F. tularensis* such as biting flies in the United States, mosquitoes the Soviet Union and ticks in Czech Republic and Austria[3]. The bacterium is known to persist in water however, directly culturable levels are below detectable levels [4]. The CDC has classified the virulent form of *F. tularensis* as a Tier 1 threat agent due to its ease of causing infection after inhaling low numbers of bacteria[5].

*F. tularensis* is a gram-negative bacterium which is the sole member of the genus *Francisella*. The genus originally contained two species *F. philomiragia* and *F. tularensis* with four species of *F. tularensis*: *F. tularensis holarctica* (Type A), *F. tularensis holarctica* (Type B), *F. tularensis mediasiatica* and *F. tularensis novicida*[5]. However,
now *F. novicida* is part of the subspecies differentiated on the basis of inability to produce acid from sucrose, ease of culturing and the lack of virulence in humans and rabbits[3]. The 16S ribosomal DNA sequences between *F. novicida* and *F. tularensis* have shown a 99.6% degree of similarity[6]. *F. philomiragia* is also included in the *Francisella* genus and is virulent only in immunocompromised individuals[7]. Despite the lower virulence, thousands of human cases occur each year and the *F. tularensis* strains can be found in environmental samples such as water or mud as well[5].

Tularemia can present with flu like symptoms, enlargement of lymph nodes and an ulcer at the site of the infection which can persist for months[8]. This form of the disease is rarely fatal with a fatality of 3%; however, an acute form of the disease called typhoidal tularemia causes septicemia and carries a mortality rate of 30 to 60% [9]. Host responses to Tularemia infections involve tumor necrosis factor produced by keratinocytes and gamma interferon produced by natural killer (NK) cells which reduces disease severity[10]. Bacteria are protected from the complement by a capsule and can invade and multiply in hepatocytes. In the highly virulent Schu S4 strain the T cells play a major role in protection during infection[3]. *F. tularensis* is an obligate intracellular pathogen of macrophages. It enters macrophages using a cytochalasin B-insensitive pathway[11].

*F. tularensis* contains a variety of virulence factors however understanding their mechanism needs further research. A significant number of genes in *F. tularensis* function in helping the bacterium survive and replicate in macrophages. Proteases and chaperons such as ClpB, ClpXP, Lon and Tig help in survival under stress.
conditions[12]. Research has shown that these proteases may help in its survival in phagosomes in macrophages. *Francisella*’s attenuated strains have genes encoding catalase (katG), DNA repair enzymes (uvrA) and cold-shock proteins (deaD)[12]. These genes may further help survival in mammalian hosts by relieving stress[12]. Protein DsbA, a lipoprotein with oxidoreductase and isomerase activity interacts with various virulence factors in the bacteria is itself a virulence factor[13]. Previous research has shown that the bacteria possesses an electron dense capsule like complex: a glycoprotein which also contributes to its virulence [14]. The *Francisella* pathogenicity island is a cluster of genes that is essential for intramacrophage replication, virulence and encodes a Type VI secretion system.

*Francisella tularensis subsp. novicida* is a less virulent subspecies of *F. tularensis* and commonly used as a model organism for the *F. tularensis*[15]. *F. novicida* (U112) has been associated with water-borne transmission of tularemia like illness in immunocompromised humans but commonly causes disease in model organisms such as mouse or *Galleria mellonella*. Both, *F. novicida* and *F. tularensis* (SchuS4) contain a gene *ftsz* which is 98% homologous. *F. novicida* has a greater than 98% identity at the nucleotide level with the human virulent strains *F. tularensis* and *F. holarctica*; however, contains only a fraction of the pseudogenes[16]. *F. tularensis* exhibits a high level of transcription of genes involved in general metabolism[16]. Genes encoding *emrA2* cluster for efflux system, *fsl* operon for siderophore production, acid phosphatase, LPS synthesis, polyamine synthesis and citrulline ureidase were highly expressed in *F. tularensis* compared to *F. novicida*[16].
There has been a lot of research conducted on *Francisella* especially trying to find new drugs or antimicrobials for the bacterium. Numerous peptides have been found to be antibacterial against *F. novicida* [17]. Cathelicidins are cationic antimicrobial peptides found in vertebrates. LL-37 is the only cathelicidin found in humans. Helical cathelicidins like *Naja atra*, *Bungarus fasciatus* and *Ophiophagus Hannah* have been discovered which inhibit bacterial growth with a broad spectrum of activity[17]. OH-CATH and NA-CATH has shown antimicrobial activity against the bacterium with higher potency than LL-37 [17]. LL-37 peptide is extremely effective against the bacterium even inhibiting the biofilm formation and has low cytotoxicity in *in vitro* assays[17]. Although antimicrobial peptides offer alternatives to traditionally used antibiotics, there still exists a need for newer antibacterial compounds.

**Curcumin**

Curcumin is a diferuloylmethane, an active compound found in *Curcuma longa* (turmeric). Turmeric has been used as a dietary spice and a coloring agent in Indian and Chinese cuisine and used to treat gastrointestinal disorders Indian and Chinese traditional medicine [18]. Curcumin is responsible for the bright yellow color of turmeric and newer research has shown that curcumin extracted from turmeric plays a therapeutic role in inflammatory disorders, cancer and in wound healing [19]. Curcumin is known to modulate a wide range of signaling molecules, which it binds to directly, such as protein kinases, inflammatory molecules, FtsZ filaments; or indirectly modulates downstream
processes[20]. It is known to indirectly target transcription factors, enzymes, inflammatory mediators and drug resistance proteins[20].

Curcumin also possess antitumor and chemo-preventive effects against a variety of cancers. It can block STAT1 and STAT3 phosphorylation as well as reduce invasiveness and migration of pancreatic cancer[21]. A study examining the efficacy of curcumin and a saffron curcumin combination showed the molecule’s effectiveness in reducing depression and anxiolytic symptoms in major depressive disorder[22]. Previous research has further proven curcumin can prevent type II diabetes, suppresses arthritis by acting as an anti-inflammatory drug[23].

Curcumin is a hydrophobic molecule with two benzene rings as shown in Figure 1 and it exhibits keto-enol tautomerism having a keto form in acidic and neutral solutions but an enol form in basic solutions[24]. This means curcumin is at a chemical equilibrium as keto and enol forms are tautomers of each other.
Previous research has shown that curcumin possesses potent antioxidant, anti-inflammatory and anti-microbial properties. It is known to inhibit the growth of various gram-positive and gram-negative bacteria as well as anecdotal and traditional use against infected wounds[25]. Curcumin is a polyphenolic compound, known to have antibacterial activity against several pathogenic bacteria including *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Enterococcus* [26, 27]. Turmeric has also shown antibacterial activity against MRSA by inhibiting MRSA invasion in human mucosal fibroblasts [26]. However, curcumin is not universally antibacterial against pathogens. Against *Salmonella typhimurium* and *Salmonella typhi*, curcumin can inhibit the protective effect of ciprofloxacin and allows proliferation of the bacteria [28]. Although curcumin is a known antimicrobial, it is hydrophobic, poorly absorbed by the cells and rapidly metabolized making its bioavailability extremely low. Curcumin conjugated with other chitosan, hexadecynoic acid and as nanoparticle formulation has shown greater antibacterial activity[29, 30].

Curcumin has been shown to reduce the biofilm formation of the gram-positive bacteria *Enterococcus faecalis*[31]. In *Vibrio*, curcumin showed inhibition of virulence factors such as the production of quorum sensing molecules. Curcumin at concentrations of 100 µg/ml reduced the number of micro colonies and disintegrated mature biofilms in *Vibrio spp*[32]. Further evidence of curcumin reducing the biofilm formation has been
shown in *Streptococcus mutans*[33]. In both cases, curcumin affected Sortase A, an enzyme responsible for the covalent attachment of Pac proteins to the cells wall in the bacterium which mediates biofilm formation[32, 33]. Some studies have also indicated that curcumin affects the integrity of gram-positive and gram-negative bacteria by distorting the shape of the bacteria and causing membrane leakage after treatment[19]. Hence, curcumin can be antibacterial in a variety of ways; however, one of the most commonly studied process of curcumin mediated bacterial growth inhibition is through it affecting the FtsZ protein.

Although curcumin possesses various benefits from antimicrobial to anticancer, anti-inflammatory properties, with low toxicity at high doses as established by various clinical trials, it has not been approved as a therapeutic agent[24]. Curcumin has poor bioavailability with low activity, poor absorption, high rate of metabolism and excretion. Furthermore, curcumin’s hydrophobicity hinders the ability of using it in culture medium. Oral administration of 1.0 g of curcumin in mouse models has shown a maximum plasma level of 0.22 µg/ml at 15min. which declined rapidly in 1h and was barely detectable in tissues[24]. Hence, curcumin has a low half-life and is rapidly metabolized which has led to a recent emergence of bioavailable variations of curcumin.

The need for bioavailable variations of curcumin arises due to growing antibiotic resistance emerging due to the overuse of antibiotics. Curcumin is effective against a variety of bacteria targeting FtsZ, membrane and even affecting biofilm in some gram-positive bacteria. Since these targets are homologous across bacterial species, curcumin may be a viable alternative to traditional antibiotics due to the emergence of antibiotic
resistance. Development of antibiotic resistance against bio-threat agents is another growing concern and the effect of curcumin against such agents needs to be assessed.

Curcumin can furthermore work with antibiotics to produce synergistic effects. Curcumin has worked with antibiotics such as cefodizime, cefotaxime and cefaclor to show synergistic effects against *S. aureus, B. subtilis, E. coli, P. aeruginosa* and *V. cholera* [34]. Curcumin has also shown synergy against *S. aureus* with antibiotics Gentamicin, Amikacin and Ciprofloxacin [35] Furthermore, previous research has also shown ciprofloxacin and curcumin work synergistically against gram-negative bacteria and may be used as a combination therapy [36]. Combination therapy of curcumin working in conjugation with other antibiotics can have a more positive effect than monotherapy with just one drug alone. Antibiotics belong to classes and target various mechanisms within the bacteria. Table 1 highlights antibiotics based on the class they’re in along with their primary target within the bacteria. Multiple antimicrobials targeting different aspect in the bacteria leads to a lowered dosage of antibiotic administered and is another alternative to traditional mono-antibiotic therapy.

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<tr>
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<td>30S ribosome: inhibits aminoacyl tRNA binding to ribosome</td>
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<tr>
<td><strong>Gentamycin</strong></td>
<td>Aminoglycoside</td>
<td>30S ribosome: protein translation, TCA cycle, Fe-S cluster synthesis, envelope formation</td>
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<tr>
<td><strong>Erythromycin</strong></td>
<td>Macrolide</td>
<td>50S ribosome: protein translation and tRNA depletion</td>
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**FtsZ**

*ftsZ* gene plays a critical role in cell division in many bacteria and encodes a 40-kDa protein that is hydrophilic and called FtsZ protein[37]. The protein is highly conserved among eubacteria with 50% amino acid identity between *E. coli* and *B. subtilis*. FtsZ protein is present in bacteria with cell walls, without cell walls and even in organisms from the Archaea domain[38]. Hence, it provides an attractive target for newer antimicrobials as previous studies have shown that mutations induced in gene *ftsZ* or protein FtsZ are lethal[37].

FtsZ protein is the cytoskeletal protein involved in cell division. It is the first protein known to localize at the cell division site where it polymerizes to form a dynamic ring structure known as the Z-ring[39]. The Z-ring formed by FtsZ may play a role as a scaffold protein to recruit other cell division proteins[39]. Previous research has shown that increasing the levels of FtsZ by two to seven fold in wild-type cells does not cause a morphological change such as an increase in the length or elongation of the cells[40].

<table>
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<td>Fluoroquinolone</td>
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Figure 2 “Dynamic behavior of FtsZ during the cell division cycle. Z ring formation is initiated from a single spot to form a ring. This process requires one minute. During division it decreased in diameter at the leading edge of the septum” Figure obtained from [41].

FtsZ protofilaments align in the middle of the cell to form the Z-ring. 30% of the available FtsZ is sequestered into the Z-ring while the remaining monomers float in the cytoplasm and exchanged with polymerized FtsZ molecules in the Z-ring[38]. The Z-ring is five to ten protofilaments thick and runs parallel to the inner cell membrane of *E. coli*. ZipA and FtsA anchor the Z-ring to the cell membrane where ZipA is a protein with a transmembrane and FtsZ binding domain; FtsA is a peripheral membrane protein binds to the cell membrane and FtsZ[42]. In *E. coli* and *B. subtilis* the Z-ring location is mediated by the Min system, MinC, MinD and MinE. Deletion of MinE leads to the filamentation of the cell and MinC prevents FtsZ polymerization[43]. There are multiple regulatory factors which mediate Z-ring formation and cell division. GTP-bound protofilaments are slightly curved and GDP bound are highly curved[38]. Hence, GTP to GDP hydrolysis
contracts the Z-ring which causes septation due to conformational changes. However, experiments have shown that the lack of GTPase activity does not prevent bacterial cell division[38]. The figure below depicts FtsZ protofilaments forming the FtsZ-ring to undergo bacterial cell division.

![FtsZ protofilaments forming the FtsZ-ring](image)

**Figure 3** Two stages of *in vitro* FtsZ GTPase reaction (left panel) and *in vivo* behavior during cells cycle (right panel). FtsZ* indicates activated form of FtsZ. The figure is taken from [44].

FtsZ is the prokaryotic homolog of tubulin family in eukaryotes. The FtsZ molecule shares 13 amino acid segments completely conserved which includes a 7 amino acid stretch almost identical to the high conserved eukaryotic α, β, γ tubulins[44]. While tubulin forms an αβ-tubulin heterodimer, FtsZ polymerizes in a monomer and *in vitro* lateral association between the protofilaments have been observed[39]. Both FtsZ and tubulin contain two domains, N-terminal and C-terminal, connected by a central α-helix
The two domains of FtsZ are β-sheets surrounded by α-helices[38]. FtsZ is a GTPase and the GTPase active site is located at the junction of two FtsZ monomer, i.e., the nucleotide-binding site of the FtsZ monomer combined with the T7 loop of another monomer[38]. There are 15,000 copies of FtsZ in *E. coli* [38]. FtsZ polymerizes in a protofilament after binding GTP, and aligns at the center of the cell. The protofilaments morphology ranges from straight to curved to other morphologies including sheets, tubes and helices. However, the straight and curved conformations of FtsZ are related to interspecies difference in domain orientation and not two interconvertible conformations[39]. FtsZ is ubiquitous hence, it is found in *Francisella* sharing homology with FtsZ found in other bacteria. *F. novicida* and *F. tularensis* both contain the homologous gene *ftsz* which produces the protein FtsZ.

FtsZ is a GTP binding protein which acts as a GTPase dependent on the concentration of FtsZ and Mg$^+$ binding as the Mg acts as a catalyst in the reaction[44]. The polymerization dynamics of FtsZ depend on its properties as a GTPase: nucleotide binding promotes longitudinal association of monomers into protofilaments while hydrolysis leads to disassembly. A single amino-acid substitution of Serine to Glycine within the tubulin signature sequence of FtsZ which is conserved in *E. coli*, *B. subtilis* and *Rhizobium melilotti* lead to the failure to initiate septum formation in cells[44].

Our hypothesis was curcumin inhibits *Francisella* growth by binding to FtsZ in *vitro* and *ex vivo*. Curcumin also caused bacterial morphological changes by binding FtsZ. Curcumin binding to FtsZ and protein ligand residue interactions were modeled and analyzed using bioinformatics software tools.
CHAPTER TWO: MICROBIOLOGY

Introduction

_F. novicida_ was used in conducting all the microbiology wet lab experiments. _F. novicida_ replicates primarily in macrophages and is adapted to an intracellular lifecycle. _F. novicida_ is typically used as a BSL II surrogate for the Tier I biothreat _F. tularensis_. There is no evidence suggesting _F. novicida_ is transmitted via vector bites and differs from _F. tularensis_ in disease symptoms [45]. However, _F. novicida_ and _F. tularensis_ share a high degree of nucleotide identity and a 92% relatedness [45]. Both _F. novicida_ and _F. tularensis_ possess the ability to infect macrophages and mimic infections as _Francisella_ is an obligate intracellular pathogen [45].

Curcumin can kill _B. subtilis_ at a MIC of 100 µM as well as heal infected wounds faster via antibacterial and anti-inflammatory mechanisms [18]. However, curcumin may also reduce antibiotic effectiveness in some cases [28] and sometimes work synergistically with other antibiotics[46]. Curcumin binds FtsZ and acts as an antibacterial in some bacteria, however it has also shown to disrupt bacterial membrane by causing cellular leakage in _S. aureus_ [19].

We _hypothesized_ that curcumin would inhibit the growth of _F. novicida_ in vitro, _ex vivo_ and _in vivo_. We conducted further assays to identify synergistic or antagonistic effects of curcumin with antibiotics as well as the mechanism of action by which curcumin prevented bacterial growth.
Methods

Chemicals

Curcumin was obtained from ACROS organics (lot: A0351590). DMSO was obtained from ATCC (lot: 30001217). Crystal Violet was obtained from ACROS organics (A0356067) Antibiotics erythromycin and gentamycin were obtained from Research Products International corp. (lot: 29670 and 32357); Doxycycline was obtained from Sigma Aldrich (lot: 1972); DiSC₃(5) from Thermo Fischer (Catalog number D306).

Bacteria

*F. novicida* (*F. tularensis novicida*) (BEI NR-13) was obtained from the Biodefence and Emerging Infection Disease Resources Repository (BEI, Manassas, VA) and *F. tularensis* B38 (ATCC 6223) was obtained from American Type Tissue Collection (ATCC), Manassas, VA. *F. novicida* was grown on Chocolate agar plates and colonies were suspended in cation adjusted Mueller Hinton II broth for a McFarland reading of 0.5. Overnight cultures were used for infection assays and frozen enumerated stocks were used for bactericidal assays.

Minimum Inhibitory Concentration

Following the NCLSI standards, MIC was performed in a low-evaporation 96-well plate with cation adjusted Mueller Hinton II broth (CAMHBII) with 2% IsoVitalex and varying concentration of curcumin, inoculated with 1 x 10⁵ CFU/ml *Francisella*
*F. novicida*. Curcumin serially diluted in 1% DMSO to various concentrations and controls (0 mg/ml no curcumin control) were added to each well. Bacteria were incubated 24 hours at 37°C without shaking. Bacterial culture densities were read at O.D. 600 nm. MIC is defined as the first clear well in the concentration curve. This was repeated 3 times with 6 replicates each time

**Biofilm Formation**

Following the MIC protocol, bacterial was grown and fluorescence taken at 24 hours, the plate was then processed for Biofilm formation using the Crystal Violet (CV) method. Briefly, the bacterial culture is gently removed from the wells, the biofilm is fixed with a methanol solution, CV is added to stain the biofilm, the wells are washed vigorously, and the remaining color is solubilized to be read at OD 570 nm [5, 15, 17, 47, 48].

**Kirby-Bauer Disc Inhibition Assay**

Standard protocol was followed [49]. Briefly, fresh colonies of *F. novicida* were inoculated in CAMHBII till it reached a MacFarland standard of 0.5. The bacteria were spread on a TSAC plate and pre-soaked discs containing various concentrations of curcumin were placed on the plate. Gentamycin (2.5 µg/ml), DI water and DMSO (1%) were used as the controls. The plates were incubated at 37 °C for 24 hours and the zone of inhibition was measured.
**Synergy Assay**

Checkerboard assay method was used for synergy experiments with curcumin and antibiotics. Briefly, in multiple 96-well plate, 50 µL of curcumin in 1% DMSO (8 µg/mL) and 50µL of different antibiotics were added to CAMHB at sub-MIC concentrations. Thus, each well had a different sub-MIC concentration of curcumin and antibiotics. 50 µL *F. novicida* was added at the concentration of 1 x 10⁵ CFU/mL. The plates were incubated at 37°C for 48 hours without shaking and the OD at 600 nm was measured.

**Francisella ex vivo model of efficacy [50]**

J774A.1 mouse macrophages (American Type Tissue Collection (ATCC), Manassas, VA) were used to assess intracellular efficacy of our compounds. The cells were cultured in DMEM supplemented with 10% fetal bovine serum. Bacteria were added to 10,000 cells per well in a 96-well tissue culture plate at a multiplicity of infection of 500 CFU per cell. The plates were then placed at 37°C, 5% CO₂ to incubate for 3 h. Supernatant was then removed; plate washed once with 200 µl PBS and 10 µl of 0.05 mg/ml gentamycin in complete media added. Plates were then incubated for 1 h and washed 2 times with 200 µl PBS. Curcumin in 1% DMSO was then added to each well at 2, 4, 8, 16, 32, and 64 µg/ml in media in triplicate. Untreated, 1% DMSO and 10 µg/ml Levofloxacin were the controls. Plates were incubated for 24 hours. Cells were washed 3 times with 200 µL PBS and 100 µl sterile dH2O added to each well. Each well was
thoroughly mixed to ensure complete cell lysis. Lysates were then serially diluted 1:100 and inoculum plated from the $10^{-4}$, $10^{-5}$ and $10^{-6}$ dilutions onto chocolate-agar plates. Plates were incubated for 24 h at 37°C. Colonies from each plate was counted and CFU/ml lysate was calculated.

Caterpillar Infection Assay

*G. mellonella* caterpillars (wax worm caterpillars) were purchased from Vanderhorst Wholesale (St. Mary’s, OH). All injections were done into the first proleg. The wax worms used weighed between 0.23-0.38 grams. Tuberculin 0.5 mL needles were used to inject the wax worms. The wax worms were stored in plastic Tupperware containers at 37°C. *Francisella* infection: 5 groups of wax worms were injected with 10 µL each of *F. novicida* (Stock = $1.5 \times 10^8$ CFU/ml, thus $1.5 \times 10^6$ CFU per caterpillar) and then incubated for 2 hours at 37°C. Following the 2-hour incubation to establish the intracellular infection, antibiotics or Curcumin or PBS were injected into the other proleg. Another dose was administered at 12 h after infection. DMSO 1% was also used as control. The caterpillars were monitored for survival by daily examination. Data analysis was performed using the Kaplan-Meier survival statistics approach on GraphPad Prism.

DiSC$_3$(5) Membrane Permeability Assay

The membrane activity of test compounds against *Francisella* were determined using the fluorescent dye DiSC$_3$(5) (3,3’Dipropylthiadicarbocyanine Iodide). [51] Briefly, 1 mg/mL solution of DiSC$_3$(5) was prepared in DMSO. It was followed by 1:100
dilutions in PBS. Bacteria was diluted to $4 \times 10^7$ CFU/mL in the PBS, DiSC$_3$(5) solution. In a black 96 well plate, 100 µL of bacterial solution was added in each well and incubated in a fluorometer at 622 excitation and 670 emission until the plate was quenched. After quenching, 100 µL of curcumin at different concentrations was added and monitored to obtain fluorescence readings. GraphPad prism was used to graph mean fluorescence of bacteria.

*qRT-PCR offtsZ in curcumin treated F. novicida*

Briefly, bacteria were grown in CAMHB containing 15 and 7.5 µg/mL concentration of curcumin in 1% DMSO for 24 hours. RNeasy mini kit (QIAGEN) was used to extract RNA from the bacteria grown in sub-MIC concentration. Purified RNA was measured using a nano drop and cDNA synthesis was conducted using standard protocol from the cDNA kit qScript XLT cDNA SuperMix from Quanta Biosciences. PerfeCTa SYBR Green FastMix from Quanta Biosciences was used for the qRT-PCR following the standard protocol from the kit. The primers were created using Thermo Fischer’s OligoPerfect primer Designer tool. The forward and reverse primers used were 5’GGTATGGGTTGTTACAGG3’ and 5’GGTCTTCAAAGGGAAAGG3’.

*Transformation and Protein Induction*

Competent *E. coli* cells were transformed with vector pDEST17 containing *ftsZ* gene from *F. tularensis* SchuS4. Competent cells used were XL1- Blue Competent Cells, Stratagene, Catalog: 200249 and 1.7 µl of 1.42M β-mercaptoethanol was added after
thawing. 1 µl of plasmid DNA was added to thawed competent cells, vortexed briefly and incubated for 30 min. The cells were heat shocked at 42°C for 45 seconds and placed on ice for 2 min. The cells were added to 0.9 ml of LB broth and shaken at 37°C for 1h at 230 rpm. 100 µl of culture was placed on LB agar plate containing 100 µg/ml ampicillin and incubated overnight. A few colonies were picked from the plate and added to LB broth containing antibiotic and incubated on a shaker at 37°C overnight. This was diluted to 1:100 in 2 ml of LB broth with 100 µg/ml Ampicillin and incubated until OD$_{600}$ was between 0.5 to 1.5. 1 ml of this culture was centrifuged at 8000 rpm and pellet saved as a control without protein induction. A 1 ml solution of LB broth with Ampicillin and 1 mM IPTG (isopropyl-1-thio-β-D-galactopyranoside) was prepared and warmed to 37°C. This was added to the 1 ml left over culture and incubated for 3h for protein induction. After 3 hours, 1 ml of the culture was centrifuged and pellet was saved for lysis and protein isolation.

*Lysis and His-Tagged Isolation and Pulldown*

The bacterial pellet was suspended in 100 µl of B-PER reagent obtained from Thermo Scientific (Catalog: PL207561) and shaken for 10min at room temperature. The solution was centrifuged at 27,000 xg for 15min to separate soluble and insoluble proteins. Some samples of the protein were sonicated to lyse the bacteria as well. Dynabeads obtained from Thermo Fisher (Catalog: 10103D) were used for His-tagged purification. Dynabeads were re-suspended in the vial by vortexing for 1min and 50 µl of Dynabeads was transferred to a microcentrifuge. The sample was prepared in 1x
binding/wash buffer (100 mM of Sodium Phosphate at pH8.0, 600 mM NaCl and 0.02%
Tween -20) and mixed. This was incubated on a roller for 5min at room temperate and
placed on a magnet for 2min and the supernatant was discarded. The beads were washed
4 times with 300 µl of Binding/Wash buffer and re-suspended after each washing step.
100 µl of His-Elution Buffer (300mM Imidazole, 50mM Sodium Phosphate, 300 mM
NaCl and 0.01% Tween-20) was added and incubated for 5min at room temperature. This
was applied on the magnet for 2min and the supernatant transferred to a clean tube. An
SDS-PAGE gel was run on the purified protein to assess the purity as described
previously[52]. Using a bioinformatics tool Expasy (http://web.expasy.org/compute_pi/),
the theoretical molecular weight and isoelectric point (pl) was calculated. Protein
molecular weight was calculated by making a linear graph of the Rf (migration distance
of protein/migration of dye front) against the logarithmic values of molecular weight of
the protein ladder standard. The unknown weight was calculated using the linear
regression equation. A Bradford assay using Bradford reagent (Sigma Aldrich B6916)
was used to measure the concentration of the protein using bovine serum albumin protein
standards.

GTPase Assay

GTPase Glo assay was obtained from Promega (Catalog: V7681). Using the
instructions manual standard protocol 4.A. for intrinsic GTPase activity protocol and 7.D.
for test compound screening using GTP Glo was used. Briefly, dilutions of FtsZ protein
and a 2X solution of GTP was made with 10µM GTP and 1mM DTT in GTPase/GAP
buffer. 12.5 µl of 2X GTP solution and 12.5 µl of GTPase was added to each well and incubated for 60-90 minutes. Then, 25 µl of reconstituted GTPase-Glo (2 µl GTPase Glo reagent 500x, 0.5 µl 10 mM ADP and 998 µl GTPase-Glo buffer) was added to the wells and incubated for 30min at room temperature with shaking. 50 µl of detection reagent was then added to the plate and it was read using Promega Glomax reader using built-in protocol for GTPase Glo assay to measure luminescence. 100 µg/ml of FtsZ protein was initially used to assess GTPase activity followed by 155 µg/ml. FtsZ was used with low concentration of MgCl₂ (10µM), as a buffer to observe change in RLUs. This was followed with using two samples of FtsZ with a buffer of Tris (50mM), MgCl₂ (5mM) and KCl (250mM) to assess intrinsic GTPase activity [53].

Results and Discussion

Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) of Curcumin was 16 µg/ml. No growth of Francisella novicida was observed at concentration of 16 µg/ml and over. The fluorescence measured had to be corrected using a broth and curcumin color only control and subtracted from the experimental OD measured. The minimum inhibitory concentration was used as a base for further Synergy and ex vivo studies.
Figure 4. Minimum inhibitory concentration of *F. novicida* in the presence of curcumin is 16 µg/ml. Growth was seen in DMSO (1%) which was used as an organic solvent to dissolve curcumin.

*Biofilm Assay*

*Francisella novicida* biofilm formation was not affected by curcumin. Biofilm was inhibited at concentrations at and above the MIC; however, there was no significant reduction in the biofilm at sub-MIC concentrations. The inhibition seen at the concentrations above the MIC was due to the inhibition of growth. Curcumin inhibited the growth of *F. novicida* and may have been bactericidal preventing the bacteria from forming any biofilm. The lack of biofilm was not due to curcumin’s effect on the biofilm production of *F. novicida.*
Figure 5. Biofilm inhibition was seen above MIC concentrations. No biofilm inhibition seen at sub-MIC concentrations as curcumin did not hamper biofilm formation.

*Kirby-Bauer Disc Inhibition Assay*

Curcumin could inhibit the growth of *F. novicida* in the Kirby Bauer disc inhibition assay with a zone of inhibition of 5 mm (100xMIC) and 13.5mm (200xMIC). However, the zone of inhibition using curcumin (100x) was not significantly different from the positive control with a p-value of 0.095 but the zone of inhibition was significantly different with a p-value of 0.019 for the concentration of 200x. But both the zones were smaller than the positive control. Curcumin did not inhibit bacterial growth better than Gentamycin on a TSAC plate. Curcumin has low bioavailability and extreme hydrophobicity most likely prevented the drug from migrating within the agar since the agar’s primary composition is water. Hence, the low migration may have resulted in the smaller sized zone of inhibition seen even at high concentrations of curcumin.
Table 2 Zone of inhibition measured at 200x and 100x MIC concentration of curcumin

<table>
<thead>
<tr>
<th>Concentration of drug</th>
<th>Zone of inhibition (mm)</th>
</tr>
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<tbody>
<tr>
<td>Curcumin (µg/ml)</td>
<td></td>
</tr>
<tr>
<td>16.0</td>
<td>0</td>
</tr>
<tr>
<td>160</td>
<td>0</td>
</tr>
<tr>
<td>320</td>
<td>0</td>
</tr>
<tr>
<td>1600</td>
<td>5 ± 7.07</td>
</tr>
<tr>
<td>3200</td>
<td>13.5 ± 1.41</td>
</tr>
<tr>
<td>Gentamycin (µg/ml)</td>
<td>18.8 ± 2.68</td>
</tr>
<tr>
<td>2.50</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>0</td>
</tr>
<tr>
<td>1%</td>
<td></td>
</tr>
<tr>
<td>Deionized water</td>
<td>0</td>
</tr>
<tr>
<td>100%</td>
<td></td>
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</tbody>
</table>

Synergy Assay

Curcumin has shown synergistic behavior with some antibiotics against gram-positive and gram-negative bacteria [36]. Curcumin and antibiotics from various classes were tested against *F. novicida* in a checkerboard assay. Curcumin worked synergistically with Doxycycline, Erythromycin and Ciprofloxacin (FIC < 0.5). Curcumin showed an additive effect with Clindamycin and Gentamycin (0.5 < FIC < 1) and no effect with Azithromycin (FIC = 1.125).

Table 3 Synergy assay conducted with various antibiotic and curcumin affecting different processes in *F. novicida*. A fractional inhibitory concentration (FIC) below 0.5 indicates
synergy, between 0.5 and 1.0 indicates an additive effect, no effect between 1 through 4 and antagonist for FIC greater than 4 [54].

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Antibiotics combined MIC (µg/ml)</th>
<th>Curcumin combined MIC (µg/ml)</th>
<th>Curcumin FIC</th>
<th>Fractional inhibitory concentration (FIC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxycycline</td>
<td>0.125</td>
<td>16</td>
<td>2</td>
<td>0.375</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>8</td>
<td>16</td>
<td>2</td>
<td>0.375</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.002</td>
<td>16</td>
<td>2</td>
<td>0.375</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>8</td>
<td>16</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>0.125</td>
<td>16</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>0.125</td>
<td>16</td>
<td>2</td>
<td>1.125</td>
</tr>
</tbody>
</table>

Francisella ex vivo model of efficacy

J774A.1 cells infected with F. novicida and treated with curcumin showed dose dependency with a significant (P-value < 0.05). There was no significant difference in bacterial load between 8 µg/ml and increased curcumin concentrations. A slight but not significant increase in bacteria was observed at 64 µg/ml. This may be due to a threshold of curcumin concentration which can enter the infected macrophages. However, sub-MIC concentrations of 8 µg/ml showed significant reduction in bacterial load.
Figure 6. Effect of Curcumin in inhibiting *F. novicida* inside J774A.1 mouse macrophages. Curcumin significantly (p-value< 0.05) inhibited grown of *F. novicida* inside the macrophages at sub-MIC concentrations. DMSO 1% showed no significant reduction in bacterial load. Levofloxacin (10 µg/ml) was the positive control showing significantly high inhibition.

*Caterpillar Infection Assay*

*G. mellonella* caterpillars were infected with *F. novicida* and then treated with different concentrations of curcumin. Even at high concentrations of 320 µg/ml curcumin did not rescue waxworms with significance when compared to the PBS treated control. Levofloxacin (10 µg/ml) rescued 100% of the caterpillars infected with the bacteria after 48h.
Figure 7. *Galleria mellonella* survival after treatment with curcumin at varied time points. No significant survival was seen after 48 h of treatment. PBS and Levofloxacin (10 µg/ml) were used as the negative and positive control showing none and complete survive of *G. mellonella*. The triangles indicate time (2h and 12h) administration of curcumin.

**DiSC₃(5) Membrane Permeability Assay**

Curcumin permeabilizes the bacterial membrane in *S. aureus*, a membrane permeability assay was conducted to assess its effect in *F. novicida*. DiSC₃(5) is a probe which accumulates in hyperpolarized membranes and translocated to the lipid bilayer. A decrease seen in fluorescence indicates an increase in membrane potential (hyperpolarization). Curcumin showed a reduction in fluorescence, however, the hyperpolarization could have been due to a false reading due to curcumin’s orange color or interference with the probe. Hence, a no bacteria control was conducted. The results of the no bacteria control were significantly different from the experimental indicating
interference due to curcumin. LL-37 significantly disrupted the membrane causing increased fluorescence due to the leakage of the dye with a p-value of 0.0095 compared to the negative control. DMSO 1% can have a positive charge on the sulfur which may have resulted in the slight increase in the fluorescence however, it was not statistically significant with a p-value of 0.2520.
B. Curcumin color only, no bacteria control showing reduced fluorescence

Figure 8. A. Membrane permeability assay showing reduced fluorescence in *F. novicida* after curcumin treatment. Curcumin hyperpolarized the bacterial membrane indicating no disruption occurred. LL-37 depolarizes the bacterial membrane showing increased fluorescence. B. Curcumin color only, no bacteria control showing reduced fluorescence

**qRT-PCR of ftsZ in curcumin treated *F. novicida***

A qRT-PCR was conducted with *F. novicida* treated with curcumin to assess the effect of curcumin on the activity of the gene *ftsZ*. The RNA obtained from the curcumin treated bacteria was assessed using a nanodrop. There was a 4.5-fold change with the lower concentration of curcumin (7.5 µg/ml) compared to a 10-fold increase at the sub-MIC concentration of 15 µg/ml. Hence, it can be hypothesized that curcumin bound to FtsZ protein which provided additional stress on the gene *ftsZ* to increase FtsZ production as seen in the increased fold change at higher concentrations of curcumin.
Figure 9. A. Fold change in \textit{ftsZ} gene after treatment with 15 and 7.5 µg/ml curcumin shows an increase in FtsZ production by the gene at higher concentrations of curcumin. Fold change calculation conducted using the ΔΔCt method. B. Concentration and 260/280 ratio of bacterial RNA purified using RNeasy kit indicating pure RNA

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
Curcumin (µg/ml) & RNA (ng/ml) & 260/280 ratio \\
\hline
0 & 297.6 & 2.10 \\
15 & 414.7 & 2.12 \\
7.5 & 338.3 & 2.11 \\
\hline
\end{tabular}
\end{table}

Protein isolation and Bradford Assay

FtsZ isolated and purified from competent \textit{E. coli} cells was used to conduct a GTPase assay to confirm that curcumin binds to FtsZ protein which lead to the increased fold change as seen in the qRT-PCR. Initially bioinformatics tools were used to estimate the theoretical molecular weight and pI of the protein estimated to be ~40kDa with a pI of 4.76 indicating it is an acidic protein.
### Figure 10. Bioinformatics analysis of FtsZ (UniProt ID: Q9ZAW3) using Expasy to estimate the theoretical pI and molecular weight.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Theoretical pI/Mw (average) for the user-entered sequence:</th>
</tr>
</thead>
<tbody>
<tr>
<td>MFDNNDMVMS NAIKKVGSVG GGGGNAVQHM CEEVSDVEFF ALNTDGQLAS KSKVQNILQI</td>
<td></td>
</tr>
<tr>
<td>GTNLTKGLGA GANPEIGKRA ATEEDRAKIEQ LLEGADMVFI TAGMGGGTGT GGAPVVAEVA</td>
<td></td>
</tr>
<tr>
<td>KEGILTVAV VTPPPFEERG RRMKAALEOQI DELTKHVDSE ITVPNEKLLS VLGKGAELID</td>
<td></td>
</tr>
<tr>
<td>AFNAANDVILG NAVGVSIEI TKPGLNVIDF ADVRAVMMNM GLAMMGGQEA SGENRAREA</td>
<td></td>
</tr>
<tr>
<td>EAAISSPILLE DINLDGAKGV IVNIAGMDM SIEFEVVEGE VIRSFISDEA IVIAGTVIPD</td>
<td></td>
</tr>
<tr>
<td>DMSDSMKIVV VVTGIKMQM KRFGVESKTS SQQSASSFS NKTSAFLRK ETEVVGASN</td>
<td></td>
</tr>
<tr>
<td>APKTDSDDDN KSDIPSSSR R</td>
<td></td>
</tr>
</tbody>
</table>

Theoretical pI/Mw: 4.76 / 39659.13

An SDS-PAGE gel was used to run the His-tag isolated purified protein from transformed *E. coli* bacteria. The calculated protein weight was 45kDa.
Figure 11. A. SDS-PAGE gel showing FtsZ protein purified in lane 1 and 2. Two ladder controls used to calculate the migration distance of the protein. The calculated protein weight was ~45kDa. B. $R_f$ represents the relative migration distance of the protein/migration of the dye front against the log of molecular weight in kDa. Linear regression equation was calculated as $Y = -202593X + 2.3595$, $R^2 = 0.921$. 

\[ \text{Rf (mm)} \]
A Bradford assay was conducted on the purified protein with bovine serum albumin. Using Bradford assay standard linear regression, the concentration of the 2 samples of protein were calculated to be 117 µg/ml and 155 µg/ml and this concentration was used to calculate 100 µg/ml concentration of FtsZ in Tris/MgCl$_2$/KCl buffer.

Figure 12. Bradford Assay standards curve with linear regression $Y = 0.2695X + 0.4741$ and $R^2 = 0.8807$.

**GTPase Assay**

GTPase assay conducted on Schu S4 FtsZ purified and isolated before. The GTPase assay was conducted to confirm that FtsZ is a GTPase and hydrolyzes GTP to GDP during cell division process. FtsZ intrinsic GTPase activity showed an increase in luciferase luminescence with Tris/MgCl$_2$/KCl buffer compared to no protein and FtsZ in MgCl$_2$ buffer.
Figure 13. Luminescence observed in FtsZ SchuS4 by GTP hydrolysis

Discussion

Curcumin inhibited the growth of *F. novicida* at the MIC of 16 µg/ml but it did not inhibit biofilm. It is possible that curcumin bound to FtsZ in *F. novicida* prevented cell division and caused bacterial cell death as a 4.5 and 10-fold change was observed in gene *ftsZ* at sub-MIC concentrations. This increase indicated that curcumin, at increased concentrations, binds protein FtsZ and stresses the gene *ftsZ* to increase protein production to continue bacterial cell division. Curcumin significantly inhibited *Francisella* growth in murine macrophages at sub-MIC concentrations. Curcumin likely
gets concentrated in the macrophages leading to a higher concentration inside the cell than the dose administered which resulted in inhibiting *F. novicida* growth.

Although previous studies have shown curcumin to eliminate infections when applied as a topical curcumin did not rescue wax worms after infection [25]. Curcumin has been shown to have poor bioavailability and low half-life in mouse models. This poor bioavailability could be a possible reason for the lack of wax worm survival. Hence, curcumin may have been eliminated from the *in vivo* model faster than the time needed to rescue the caterpillars from *F. novicida* infection. It possibly works as a topical agent on the skin but inside the organisms may get metabolized faster than required for survival after infection inside the organism.

Curcumin’s hydrophobic nature lead to a small zone of inhibition compared to the positive control. Curcumin molecules can kill *F. novicida in vitro* at 16 µg/ml but the molecule was not able to migrate through the hydrophilic agar to prevent bacterial growth. Hence, high concentrations of curcumin were required to create a zone of bacterial growth inhibition. Using a hydrophilic form of curcumin would create a larger zone at lower concentrations of curcumin.

Curcumin and antibiotics target different systems in the bacteria which resulted in the synergistic and additive effects. Synergy was seen with doxycycline and erythromycin, both antibiotics inhibit tRNA preventing the translation of mRNA to proteins, inhibiting protein formation. This was synergistic with curcumin known to bind FtsZ protein and as this study showed, increased *ftsZ* gene fold change. Hence, curcumin may stress the cell to increase protein production and the antibiotics bind tRNA may
explain the synergy seen with these antibiotics. This was also true of the additive effect seen with Clindamycin and Gentamycin as the former targets protein translation and the latter, TCA cycle, Fe-S cluster and envelope formation. No effect was observed with Azithromycin but synergy seen with Erythromycin even though both belong to the class macrolide of antibiotics. One possible reason for this could be the longer half-life associated with azithromycin which makes it distinct from other macrolides [55].

Curcumin is known to have a shorter half-life which contributes to its low bioavailability [24]. Hence, the differences in half-life could potentially contribute to the lack of effect observed with azithromycin as opposed to erythromycin. Curcumin may have been processed by the cell while azithromycin was targeting its bacterial components and the antimicrobials were not in the bacteria at the same time possibly due to different half-lives.

Curcumin also has a bright orange color which interferes with many fluorescence measuring assays. The color has a wavelength of 590-620 nm which is within the range used in microbiology assays. A color control of curcumin was used in every assay to eliminate color interference however, DiSC₃(5) assay showed reduction in luminescence due to the interference. This reduced fluorescence as well as the qRT-PCR results, eliminated the possibility of membrane permeabilization hence, we can conclude that curcumin did not permeabilize the membrane of *F. novicida*.

A GTPase assay was conducted to confirm curcumin binding FtsZ. However, the GTPase assay showed an increase in luminescence while measuring intrinsic GTPase activity of FtsZ from *F. novicida*. FtsZ is a GTPase which hydrolyzes GTP to GDP. The
GTPase assay measures GTP left in the well by converting it to ATP to measure luciferase activity. However, adding FtsZ with buffer showed increased RLUs compared to no protein control. The increase may have been due to interference. FtsZ purified using His-tag may have had GTP bound to it which caused increased luminescence. Furthermore, the buffer, used for GTPase assay based on a previous study, may have interfered with the experiment causing the increase in luminescence. This assay needs more reagents to activate FtsZ protein as well as a positive control FtsZ from E. coli to confirm intrinsic GTPase activity and then further test curcumin inhibiting GTPase activity of FtsZ.

Overall, curcumin showed it killed F. novicida in vitro as well as ex vivo but its poor bioavailability prevented rescuing in vivo. Curcumin also showed synergy and additive effects with antibiotics which lead to using sub-MIC concentrations of the drugs to eliminate the bacteria. Future experiments with a bio-available form of curcumin need be conducted to rescue model organisms after infection. Curcumin nano-particles or PEGylated forms of curcumin are typically hydrophilic with low toxicity and not easily metabolized by living organisms[56]. These can be used for more conclusive results while exploring the mechanisms of bacterial inhibition by curcumin.
CHAPTER THREE: MORPHOLOGY

Introduction

Curcumin has previously shown to increase GTPase activity of FtsZ and inhibit its assembly. Treatment with curcumin can affect bacterial morphology. Previous studies have shown curcumin causes filamentation in *B. subtilis* by binding FtsZ and preventing septum formation in bacterial cell division[57]. Curcumin is an FtsZ inhibitor which leads to filamentation and eventually cell death in bacteria[38]. Bacterial morphology can be visualized by staining the bacteria and observing under a light microscope.

Dynamic Light scattering is also known as photon correlation spectroscopy. It measures the size of particles in the range of 0.5nm to 10µm[58]. DLS measures the movement of scattering particles by measuring the frequency shift of the incident laser light when scattered by motion inside the solution. The doppler effect due to the Brownian motion of the particle determines the mean size[59]. However, particle dust, protein interactions and ligands can cause inaccurate readings using the DLS.

Flow cytometry is a technique which uses fluidics system and laser optics for fluorescence detection. Flow cytometry analysis can provide estimates on particle size based on forward scatter measurements[60]. Cytometry is typically used to label cells of interest and identity fluorescent intensity and cellular morphology[60]. We tested *F. novicida* treated with curcumin to assess changes in bacterial morphology using microscopy, dynamic light scattering and flow cytometry.
Methods

Microscopy

Curcumin treated *F. novicida* was gram stained. Bacteria were grown in 15 µg/mL and 8 µg/mL concentrations of curcumin for 24 hours at 37°C in a shaker. 10 µL of bacteria was placed on a slide and standard gram staining protocol was used. Briefly, the specimen was heat fixed on a flame and flooded with crystal violet solution for 1 minute. After rinsing with distilled water, iodine solution was added for 1 minute, followed by rinsing and decolorizer for 3 seconds. After the decolorizer was rinsed, safranin was added for 30 seconds and rinsed. The slide was left to dry for 4 hours and observed using a light microscope at 100x, 400x and 200x resolution.

Dynamic Light Scattering

Bacteria was grown CAMHBII from a fresh colony in a 15mL tube at 37°C with shaking until the McFarland standard of 0.5 was reached. Bacteria was incubated with different concentrations of curcumin (0 µg/mL, 8 µg/mL, 16 µg/mL, 32 µg/mL, 64 µg/mL and 1 mg/ml) for 2h. Approximately 200 µl of bacteria was placed in a cuvette and DI water added for analysis. The cuvette was shaken and cleaned to ensure no dust particles or bubbles remained and placed in the DLS reader for analysis.

Flow Cytometry

Bacteria was grown in CAMHBII from a fresh colony in a 15mL tube at 37°C with shaking until the McFarland reading between 1 and 2 was reached indicating a cell
density between 3 and $6 \times 10^8$ CFU/ml. Bacteria was incubated with varied concentrations of curcumin (0 µg/mL, 2 µg/mL, 4 µg/mL, 8 µg/mL, 16 µg/mL and 32 µg/mL), DMSO 1% and curcumin color only controls were also used. Bacteria was incubated for 4 and 8h and were centrifuged to obtain a pellet. The pellet was gently mixed in a solution of 5 ml of 1% paraformaldehyde in dPBS to fix the bacteria. After 30 minutes of fixation, the bacteria were centrifuged and pellet washed 3 times with 5 ml dPBS. The final pellet was mixed gently with 3 ml dPBS and stored at 4°C. 1 ml of the solution was aliquoted for cytometry analysis. BD FACsAria II obtained from BD Biosciences was used for FACs analysis.

**Results**

*Microscopy*

*F. novicida* treated with curcumin showed no filamentation when observed under the microscope. Bacteria was observed using light microscopy after gram staining. However, sub-MIC concentration of curcumin did not induce visible filamentation in *F. novicida*. 
**Dynamic Light Scattering**

DLS showed a significant increase in mean size scattering of the particles in the sample. The mean showed average particle diameter or Z-average size. The PDI indicated the polydispersity index where high PDI (PDI > 0.7) indicates a broad size distribution. In *F. novicida* treated with curcumin we observed an increase in mean diameter of the size. However, the high PDI indicated that the bacteria had a large size distribution. Furthermore, the percent error was high. This high percentage indicates a high likelihood the mean size obtained was by chance. Although there was a significant increase in
particle size after curcumin treatment, the high PDI and error percentage may indicate inaccuracy in the data.

Table 4 Mean particle size obtained by dynamic light scattering with polydispersity index and error percentage. PDI < 0.7 indicates broad particle size range. Error > 5% indicates high probability mean size obtained by chance

<table>
<thead>
<tr>
<th>Concentration of Curcumin (µg/ml)</th>
<th>Mean size (nm)</th>
<th>Polydispersity Index</th>
<th>Error percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>651.85</td>
<td>1.452</td>
<td>0.56</td>
</tr>
<tr>
<td>8</td>
<td>1940.37</td>
<td>1.394</td>
<td>21.76</td>
</tr>
<tr>
<td>16</td>
<td>1272.9</td>
<td>1.343</td>
<td>3.14</td>
</tr>
<tr>
<td>32</td>
<td>1974.5</td>
<td>1.58</td>
<td>27.46</td>
</tr>
<tr>
<td>64</td>
<td>971.9</td>
<td>1.168</td>
<td>4.86</td>
</tr>
<tr>
<td>1000</td>
<td>4309.9</td>
<td>0.844</td>
<td>65.45</td>
</tr>
</tbody>
</table>

Flow Cytometry

There was a significant increase in bacterial granularity seen after curcumin treatment. SSC-A used to measure the changes in internal complexity showed a dose and time dependent relationship on bacteria treated with curcumin. Increased granularity was seen at higher concentrations of curcumin. This increase was also seen as the incubation time increased. Curcumin bound to FtsZ prevent bacterial cell division however, the bacteria can continue producing proteins and nuclear material for cell division which
could explain the increase in granularity seen after treatment. No change in the actual size of the bacteria was seen using the FSC-A parameter.

![Graph showing relative fluorescence intensity (SSC-A) against concentration of curcumin (µg/ml)](image)

**Figure 5. Dose dependent increase in fluorescence seen at 4 hours of treatment**

**Discussion**

DLS showed an increase in the diameter and length of bacterial cells after curcumin treatment. However, no filamentation was observed under a microscope.

Curcumin has shown to bind FtsZ in *B. subtilis* causing filamentation. In *F. novicida* we can conclude curcumin binds FtsZ preventing septation since an increase in bacterial size was observed. However, the bacteria may not have survived long enough to filament after curcumin treatment. Furthermore, *F. novicida* is a small bacterium with the size, 0.2-0.7
μm diameter compared to 4-10 μm of B. subtilis. The smaller size may have made it harder to observe filaments formed with F. novicida. Elongation and filamentation may further have been inhibited by F. novicida proteins or bacterial cell leakage and death. Transmission electron microscopy would provide clearer images of bacteria treated with curcumin. The filamentation seen in B. subtilis but not F. novicida may be due to F. novicida’s inability to form filaments due to reduced homology between cell division proteins. This needs to be further explored to conclude if F. novicida can produce filaments.

DLS showed an increase in size indicating curcumin bound at FtsZ prevents cell division and increases bacterial size. However, the high error indicated that there were additional particles in the sample. Some bacterial cells may not have survived the treatment and leaked its proteins and cytosolic components in the media causing the high size variability and increased light scattering.

Cell cytometry was conducted to assess the increase in bacterial size. Although no significant increase in bacterial size was seen, we observed an increase in internal complexity and granularity after treatment. FACs machine is typically not designed for bacterial cells, and F. novicida’s size may be harder to detect through 0.75 nozzle. This small size could have been the reason for difficulty in observing a significant change in bacteria. SSC-A measuring the internal complexity of the bacteria showed a significant increase in dose dependent treatment of bacteria. Curcumin binds FtsZ which prevents cell division, however, the bacterial cell likely continues making nuclear material and proteins necessary for cell division. SSC-A measured the amount of content inside the
bacteria cell. Curcumin treatment showed an increase in granularity inside the cell which may be due to the bacteria continuing to make necessary material for division but unable to divide due to curcumin.

Bacterial morphology overall, was not significantly changed after curcumin treatment. No elongation or filamentation was observed. DLS showed an increase in size however, high error percentage and PDI indicated that the data may not be extremely accurate. Cytometry showed increase in granularity indicating increased proteins and nuclear material in the cell due to curcumin preventing cell division.
CHAPTER FOUR: BIOINFORMATICS

Introduction

FtsZ is a ubiquitous and essential protein found across all prokaryotes except Chlamydiae and Crenarchaea [61]. It has shown high homology across bacterial species. It is an essential protein where displacing the GTP or modelling an amino-acid substitution in FtsZ leads to failure to initiate septum formation [44]. Due to the increase in antibiotic resistance seen, FtsZ provides a target for antimicrobials. However, the exact mechanism of FtsZ inhibitors and Z-ring contraction is highly debated and computation models provide excellent methods to find the binding sites of FtsZ inhibitors.

Computational tools and bioinformatics can be used to assess the GTP binding site of FtsZ. Curcumin has known to bind tubulin and inhibit microtubules polymerization in eukaryotes [62]. FtsZ is a prokaryotic homolog of the eukaryotic tubulin family and curcumin has shown to increase GTPase activity in purified FtsZ from B. subtilis [63]. Hence, we hypothesized, curcumin would bind F. novicida FtsZ at the GTP binding site using bioinformatics tools to model the ligand binding.

The GTP binding sites and regions have been identified in F. novicida based on homology (UniProt ID A0Q4A9). Using this protein sequence, FtsZ homology was confirmed with F. tularensis, an FtsZ model for F. novicida was generated and ligands (GTP and curcumin) were docked on the protein.
Methods and Results

Homology Modelling

Emboss Needle was used to run a Pairwise Sequence alignment of FtsZ from *F. tularensis novicida* (U112) and *F. tularensis tularensis* (SchuS4) using the BLOSUM 62 matrix. The pairwise sequence alignment showed only one site altered in *F. novicida* and *F. tularensis* residue 322 changed from Proline to Leucine. Residue identity was 99.7% with a 99.7% similarity and a score of 1875.

The critical GTP binding sites identified by Uniprot using homology at 138 (E), 142 (R), 186 (N) were conserved. The GTP nucleotide binding regions from 21-25 as well as from 107-109 were also conserved.
Figure 16. Pairwise sequence alignment of FtsZ protein in F. novicida U112 and F. tularensis SchuS4 using Emboss Needle showing homology. Stars above the amino acids indicate GTP-binding critical conserved residues and lines indicate conserved nucleotide binding regions.

**FtsZ Structure Prediction**

Multiple structure prediction tools were used to confirm and predict *F. novicida* FtsZ structure. Initially the FASTA format of FtsZ (U112) was obtained and SWISS-MODEL was used to find templates to model FtsZ [64]. SWISS-MODEL predicted and template used had the PDB ID 1ofu.1.A, Crystal structure of FtsZ from *Pseudomonas Aeruginosa* with a 2.1 Å resolution obtained via X-ray Diffraction. ModBase [65] was
used to confirm model obtained on SWISS-MODEL. ModBase similarly used template 1ofu.1.A to generate FtsZ model.

![FtsZ monomer modelled on SWISS MODEL](image)

![FtsZ dimer from *P. aeruginosa* obtained using X-ray diffraction from PDB](image)

Figure 17. A. FtsZ monomer modelled on SWISS MODEL\[64\] using 1ofu.1.A template. B. FtsZ dimer from *P. aeruginosa* obtained using X-ray diffraction from PDB \[66\].

This model showed a high sequence identity of 62.89% with a 0.83 coverage. The GMQE (Global Model Quality Estimation) was 0.72 indicating high reliability. The QMEAN was -0.10 indicating that the model was of comparable quality to experimental structures. Normalized QMEAN score comparing the model with non-redundant PDB structures showed the model quality fit with experimental structures. The local quality
estimate showed two residues with local similarity below 0.6 indicating the model had high quality.

Figure 18. A. Normalized QMEAN4 z-score of predicted model is < 1 [64]. B. Local Quality Estimate of each residue of the predicted model. A score below 0.6 indicates low reliability.
Phyre2 [67] was used to confirm that the manually selected template used to create the model was reliable. Phyre2 predicted a model based on template 1ofu with 100% confidence indicating high probability the model is homologous to the template and 65% sequence identity. Hence, Phyre2 confirmed the template used to generate FtsZ model was accurate and the model created using SWISS-MODEL was further used for ligand docking.

The model was further confirmed by using a Ramachandran analysis and ProQ2. Ramachandran plot shows where the residues lie in favorable or unfavorable regions on the Ramachandran plot based on the backbone phi/psi angle. ProQ2 analyses the local and global quality of the model. Using these two analytical tools we confirm the model has good quality and stable phi/psi bonds indicating a highly reliable FtsZ model.
Figure 19. A. Ramachandran analysis of the FtsZ model indicating favorable (blue) regions as well as allowed (green) regions. B. ProQ2 quality assessment showing a range of local and global quality of the model. Good quality (red, orange) medium (yellow, green), bad quality (blue).

FtsZ Ligand Binding

Initially RaptorX [1] was used to predict the binding site residues which interact with GTP binding in FtsZ. It predicted GTP as the ligand binding to FtsZ U112 at pocket 81 with a low P-value of 1.45 e-08. The pocket multiplicity of 81 indicates the predicted GTP binding pocket of FtsZ is true and the low p-value indicates the model is of a high quality. The uGDT score indicating unnormalized Global Distance Test was 280, a uGDT greater than 50 indicates a good model. The uSeqID was 198 which is the number of identical residues in alignment. Higher uSeqID is better indicating that the predicted model has the correct fold. The predicted binding residues were:


3DLigandSite [67] server was also used to confirm the above binding sites obtained using RaptorX. The binding sites obtained using 3D Ligand Sites were based on the model created by Phyre2 based on the template 1ofu.1.1A. The binding sites were same as ones obtained using RaptorX.

The structure of curcumin was obtained on PubChem CID 969516 and GTP on PubChem CTD 6830. Chimera [68] was used to view and convert the SDF structures to PDB and mol2. Initially, PatchDock [2] a molecular docking algorithm based on shape
complementarity principles was used to dock the two ligands on *F. novicida* FtsZ model. The relative score on Patchdock was 5332 with a 630.60 area covered by the ligand.

Using Chimera, the amino acid and ligand interactions were observed:


![Image](image1.png)

Figure 20. A. Curcumin Docked on FtsZ predicted model from *F. novicida* visualized using Pymol [69] B. Amino acids (red) interacting with curcumin molecule (grey) visualized using Chimera.

Similarly, GTP protein was docked on *F. novicida* using Patchdock. The relative score of the model used was 4166 with a coverage area of 501.10. Using Chimera, the amino acid ligand interactions were as follows:

N25, T132, E138, R142, N165, F182
Figure 21. A. GTP Docked on FtsZ predicted model from *F. novicida* visualized using Pymol [69] B. Amino acids (red) interacting with GTP molecule (grey) visualized using Chimera.

The table below summarizes amino acid interactions between ligand (curcumin or GTP) on FtsZ based on various software analyses described above. E138 and R142 are FtsZ binding sites based on all the bioinformatics tools. Furthermore, Curcumin-FtsZ and GTP-FtsZ share 5 FtsZ residue and ligand interactions. This indicates a strong likelihood that GTP and curcumin bind at the E138 and R142 binding pocket in *F. novicida* FtsZ.
Table 4. Amino acid residue interactions predicted by RaptorX[1], PatchDock[2] modeling of curcumin and GTP on FtsZ and Uniprot (A0Q4A9). E138 and R142 binding sites are conserved amongst all 4 bioinformatics tools used.

<table>
<thead>
<tr>
<th>FtsZ Interactions Raptor-X</th>
<th>Curcumin FtsZ Interactions using PatchDock</th>
<th>GTP FtsZ interactions using PatchDock</th>
<th>UniProt FtsZ Homology Predicted Binding Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>G20</td>
<td></td>
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<td></td>
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<tr>
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</tr>
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<td>N43</td>
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<tr>
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<td></td>
<td>G107</td>
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<tr>
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<tr>
<td>A185</td>
<td></td>
<td>A185</td>
<td></td>
</tr>
<tr>
<td>N186</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Discussion

Using bioinformatics tools, we could create an accurate model of *F. novicida* FtsZ based on the template 1ofu.1.A from *P. aeruginosa* created by Cordell et al. The high sequence identity, coverage and GMQE indicated the model predicted was highly reliable
compared to an experimentally determined structure. The predicted model was confirmed with Phyre2 and model quality was assessed further to confirm the reliability of the structure. Curcumin and GTP ligand docking on FtsZ confirmed the two ligands binding at the same pocket on the protein. Binding sites E138 and R142 were the same using Patchdock, 3D Ligand site, RaptorX as well as UniProt homology based binding sites. T132 and N165 were also common in all but UniProt.

This modeling showed curcumin binds at the GTP binding site on *F. novicida* FtsZ. The inhibition seen in *F. novicida* growth may have been due to curcumin binding the protein and inhibiting cell division by preventing GTP hydrolysis in FtsZ. Curcumin prevents the septum formation during bacterial cell division in *Francisella*. The binding sites are homologous between *F. novicida* and *F. tularensis*. Hence, curcumin may bind in FtsZ obtained from SchuS4 at the same binding site preventing GTP binding and inhibiting bacterial growth.

This mechanism modeled using bioinformatics tools needs to be confirmed using laboratory analysis. Previous studies have confirmed curcumin displaces GTP in *E. coli* and *B. subtilis* by binding FtsZ and preventing cell division. This interaction has also been modeled using X-ray crystallography to obtain the actual FtsZ structure and docking the ligands using bioinformatics.

FtsZ poses a ubiquitous and highly homologous target for antibacterial compounds. Curcumin can displace GTP in the modeled FtsZ hence, we can further develop a bioavailable derivative of curcumin to inhibit bacterial growth. This derivative
can be further modeled to ensure it interacts with the same residues as curcumin shown in our findings and may pose a new alternative for treatment of Tularemia.
CHAPTER FIVE: CONCLUSION

There has not been a significant research conducted on the effect of ayurvedic medicine curcumin on *Francisella. F. novicida* is a surrogate used for *F. tularensis* in BSL II lab settings as it successfully infects macrophage cell lines as well as animal models while sharing high nucleotide identity with the virulent strain[45]. Curcumin, a dietary spice, has been used for centuries to treat gastrointestinal illnesses, wounds, arthritis, scabies and skin diseases. In scientific literature, curcumin has been effective in wound healing and inhibiting bacterial growth due to anti-inflammatory and anti-bacterial properties[18]. Curcumin’s mechanism of action in killing bacteria as well as synergistic effects has been previously explored against *S. aureus, E. coli, B. subtilis* and many other gram positive and negative bacteria[35, 57]. Here, we assessed curcumin’s antibacterial effects against *F. novicida in vitro*, in living cells and in model organisms. Curcumin’s synergistic ability with antibiotics and mechanism of action was also explored. Changes in bacterial morphology after curcumin treatment were analyzed and finally, bioinformatics tools were used to model *F. novicida* protein FtsZ and predict ligand binding sites to help understand curcumin’s mode of action.

We showed that curcumin kills *F. novicida* with a minimum inhibitory concentration of 16 µg/ml without affecting its biofilm. Curcumin worked synergistically with doxycycline and erythromycin but showed no effect with azithromycin. This may be explained by varied half-lives of the two drugs in bacterial cells. Both drugs may not have been present in the bacteria at the same time due to faster metabolism of curcumin.
and slower than average metabolism of azithromycin[55]. Curcumin significantly prevented bacterial infection in murine macrophage J774A.1 cells at concentrations lower than the MIC. This was likely due to curcumin concentrating inside the macrophage leading to bacterial elimination of the cell. However, curcumin was not effective in rescuing infected caterpillars. Low bioavailability would explain the failure in rescuing the model organisms. Furthermore, synergy observed with two classes of antibiotics (tetracycline and macrolides) indicate curcumin may work well in combined therapy with other antibiotics at lower concentration which may reduce side-effects associated with many antibiotics. However, this needs to be tested further in model organisms.

Bacterial morphology showed a significant increase in granularity after using flow cytometry. The increase in granularity was likely due to protein and nuclear material made to undergo cells division as the curcumin treatment prevented the cell from dividing. No filamentation was seen in F. novicida treated with curcumin unlike B. subtilis which indicates that F. novicida may not form filaments even if curcumin bound FtsZ.

Curcumin binds FtsZ in F. novicida. This was shown using bioinformatics analysis to model an accurate and reliable FtsZ from F. novicida based on an X-ray crystallography obtained template from P. aeruginosa. Curcumin bound at the GTP binding sites predicted by RaptorX, 3D Ligand Site and UniProt homology based prediction. Docking curcumin and GTP showed they bind at the same pocket and share multiple residue interactions. Curcumin experimentally showed am increased stress on gene ftsZ to increase protein production at sub-MIC concentrations of curcumin. GTPase
assay to test intrinsic activity of FtsZ as a GTPase showed an increase in luminescence in the presence of a buffer. This increase was likely due to the presence of GTP bound to FtsZ during purification or interference from a component of the buffer used during purification.

Experimental analysis needs to be conducted to examine mechanism of action but we can conclude via reliable bioinformatics methods that curcumin binds FtsZ protein in Francisella. This is an interesting observation as this has been shown not just in Francisella but also other bacterial and poses an alternative to current antibiotic therapeutics. Hydrophilic and bioavailable forms of curcumin which bind the same residues as pure curcumin in FtsZ can be created synthetically as monotherapy or work synergistically with current antibiotics. Curcumin can also prevent bacterial growth by disrupting membrane [19], has anti-inflammatory and anti-cancer properties[20]. All these effects of curcumin can help develop an antibacterial therapy as an alternative to current therapeutics. The emergence of antibiotic resistant bacteria has increased the stress to create newer antibiotics which target ubiquitous proteins and create fewer resistant bacteria. FtsZ is an excellent target for new antibiotic therapies and curcumin binding FtsZ poses a new therapeutic that needs to be further explored.
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BIOGRAPHY

Shravani Bobde graduated from Scripps college with a Bachelor of Arts in Biology and Mathematics minor. She is now completing her Master of Science at George Mason University.