Antimicrobial effects of lactoferrin and cannabidiol on *Borrelia burgdorferi*

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Abstract
Robust antimicrobial resistance in pathogenic bacteria such as *Borrelia burgdorferi*, the Lyme disease bacteria, has proven difficult to alleviate in affected patients. Due to the bacterium's ability to form biofilms in unfavorable conditions, standard means of eliminating infection has shown to be ineffective and has lead the need to find a novel biologically active compound to combat these infectious agent. Lactoferrin previously has been shown to have antimicrobial effects on biofilms formed by *Pseudomonas aeruginosa* in cystic fibrosis patients. In this project, the effect of lactoferrin was tested against the biofilm form of *Borrelia burgdorferi*. The results show that the amount of biofilm forms can be significantly reduced by lactoferrin. In summary, lactoferrin has showed promising effect on some of the forms of *Borrelia burgdorferi* and may prove to be a safe and effective treatment for patients with persistent B*orrelia burgdorferi* bacterial infections. Further research is necessary to fully describe its effect on the biofilms and the ability of them to survive after therapy.

Introduction:

Borrelia burgdorferi is the bacterium found in deer ticks which is responsible for Lyme disease. *B. burgdorferi* enters the blood stream when a tick bites a human or an animal.[1] Disease symptoms, including bull's eye rash known as erythema migrans, fatigue, chills, fever, headache, muscle aches, joint aches, swollen lymph nodes, and neurological complications can take weeks, or even years to appear.[1] Patients can experience variety of symptoms such as facial palsy and long term approximately 60% experience arthritic symptoms. Lyme disease was discovered in the 1970s in Lyme, Connecticut.[2] According to the Center of Disease Control and Prevention, approximately 300,000 people are diagnosed with Lyme disease annually in the United States.[2] Standard treatment for Lyme disease patients is antibiotic therapy but relapse is common after the therapy is discontinued. Our research group has demonstrated that standard antibiotics have a very limited effect on Borrelia biofilm.[12]

Administration of tetracyclines (e.g. doxycycline) or macrolides (e.g. clarithromycin) are the primary treatments currently being used. Three months of tetracycline and macrolide treatment results in 50-60% reduction in symptoms with a cure rate of as little as 20%.[3,4] Doxycycline, the tetracycline thought to be the best current treatment for the disease, still only results in approximately 20% of patients.[3,4,5,6] Chronic Lyme disease rarely results in a successful treatment and immune systems cannot suppress recurrence after treatment is discontinued.

The Lyme disease research group at the University of New Haven recently demonstrated that *B. burgdorferi* can form a third, very organized structure called a biofilm.[7] *B. burgdorferi* can take several forms: the spirochete, the cyst and the biofilm form. Biofilms are structured communities, encircled by a self-manufactured polymeric matrix to prevent *B. burgdorferi* from adverse environmental conditions. Biofilms make the treatment of *B. burgdorferi* extremely difficult as it can increase its resistance up to 1000 times, as opposed to individual spirochetes.[8,9] This biological defense could be the reason why Lyme disease can be so resistant and reoccurring. The compound chosen to investigate antibacterial effects on *B. burgdorferi* biofilm was lactoferrin.

Lactoferrin is a protein commonly found in cow and human milk and has shown to have antimicrobial effects against a wide range of bacteria and have been used in the medical field to combat bacterial infections.[10] Lactoferrin can be found in fluids through our body including our nose, eyes, respiratory tract, and intestines. Lactoferrin is currently a treatment for diarrhea, hepatitis C, preventing cancer, and stomach and intestinal ulcers. It is also used as an antioxidant against bacterial and viral infections.[10] Lactoferrin absorbs and sequester iron and manganese, depriving bacteria from essential nutrients. Therefore, the effectiveness of lactoferrin was tested against *B. burgdorferi* biofilms and compared to doxycycline, which is the standard antibiotic for Lyme disease treatment.

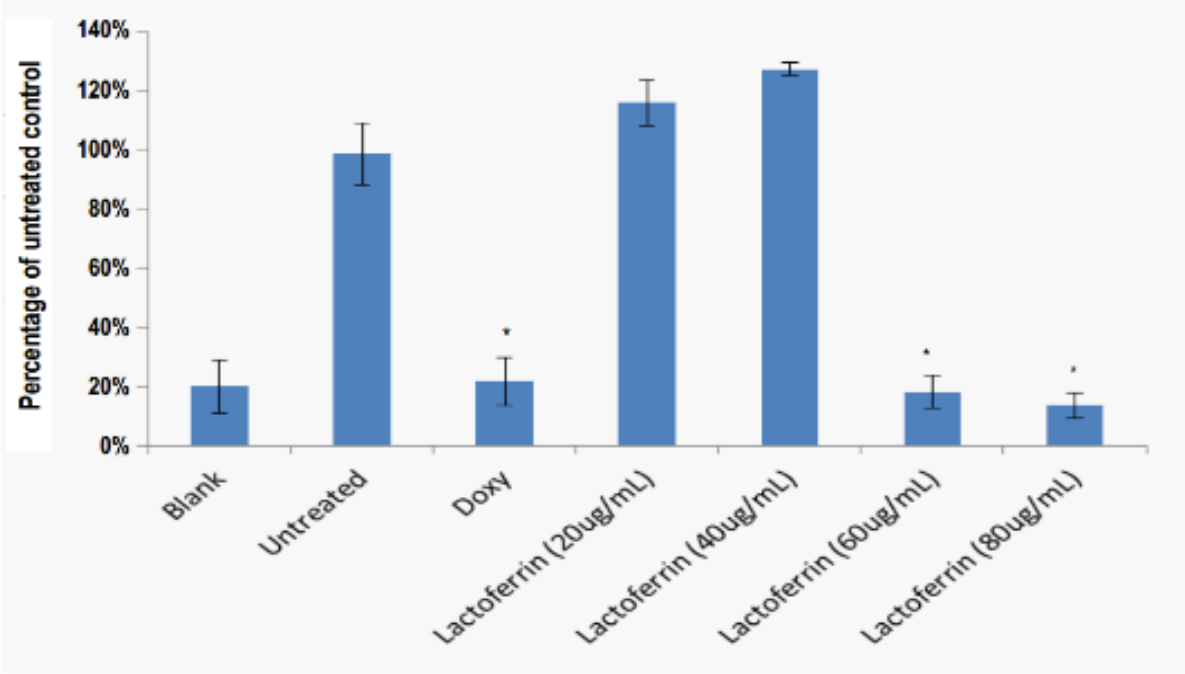

Fig 1. Quantitative analysis of *B. burgdorferi* biofilm treated for 72 hours with Lactoferrin. MTT assay was performed on biofilm of *B. burgdorferi* treated with different concentration of lactoferrin). Data was considered significant at p value<0.05. * indicates p value≤ 0.05

Materials and Methods:

Low passage isolates of B31 strain of *B. burgdorferi* sensu stricto were cultured in Barbour-Stoner-Kelly H (BSK-H) complete medium supplemented with 6% rabbit serum (PelFreeze). Serial dilutions of *B. burgdorferi* spirochetes were grown for 6-days in a 48-well plate to initiate biofilm formation, followed by incubation with a tetrazolium dye, MTT (2 mg/ml in PBS) for 4 hours at 33˚ C. After incubation, the pellet was resuspended in 150µL of isopropanol and incubated on a rotatory shaker for 15 minutes. Absorbance of the supernatant was read at 570 nm using the Eon microplate spectrophotometer.

B. burgdorferi biofilms were cultured as mentioned above and treated for three consecutive days (72 hours) with varying concentrations of antimicrobial agents and biofilm viability of Borrelia biofilms was determined using the MTT assay as described above.

As a positive control, 25 µg/ml of doxycycline (doxy), and as a negative control, the appropriate volume of PBS buffer, were used instead of antimicrobial agents. The two-sample paired t-test statistical analyses were performed using GraphPad Prism 6.00 for Mac (La Jolla, CA, USA).

Results and Discussion:

The experimental design included samples with a row of blank media, untreated *B. burgdorferi* (negative control), doxycycline (positive control) and lactoferrin in various concentrations. It was important to test various concentrations to be able to determine what amount of the antimicrobial compound could be efficient in effectively eliminating the *B. burgdorferi* biofilm. The concentrations tested were those used in literature, which varied from 20 ug/mL to 80ug/mL. Figure 1 shows that lactoferrin, at a concentration of 80 ug/mL, was the most effective and decreased the viability of *B. burgdorferi* biofilm by 15% compared to the untreated sample. Comparing to positive control, lactoferrin was ~5% more effective than doxycycline, which is a compound commonly used in hospitals for treatment of Lyme disease. Interestingly, lactoferrin at lower concentrations, such as 20ug/mL and 40ug/mL, increased *B. burgdorferi* biofilm viability compared to the untreated control, however the effects were not significant.

Originally the experiment had considered allicin, an antimicrobial compound found in garlic, to treat *B. burgdorferi* biofilms but despite use of the compound in varying concentrations from 2ug to 10ug which was the literal values found, it had no significant antimicrobial effects on Borrelia biofilms (Figure 2). Shortly after lactoferrin was chosen and tested for its antimicrobial effects.

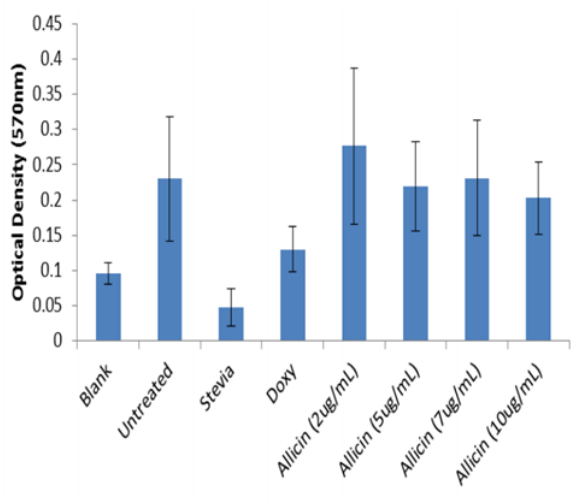

Fig 2. Quantitative analysis of Borrelia burgdorferi biofilm treated with different concentration of allicin using MTT viability assay. Negative control was a sample treated with vehicle (PBS buffer) and positive control had doxycycline (doxy) and Stevia, which was shown by previous research to have a significant effect on *B. burgdorferi* biofilm.

Conclusions and Future Work:

Data from this study showed that lactoferrin could have a significant effect on the viability of *B. burgdorferi* biofilm, while allicin did not show any potential effect. The mechanism of the antimicrobial effect of lactoferrin has been previously suggested to work by absorbing and therefore sequestering iron and manganese, so bacteria cannot utilize it. In a recent Nature publication, lactoferrin was demonstrated to significantly compromise the formation of biofilms of *Pseudomonas aeruginosa*. Lactoferrin chelated iron, disallowing *P.* aeruginosa from taking up iron, causing "twitching, a specialized form of surface motility." [10] This caused the bacteria "to wander across the surface instead of forming cell clusters and biofilms." This movement through *P. aeruginosa* generations prevented organized biofilm structures to form so cells remained in a thin layer and were later proven to lose the antimicrobial resistance associated with organized biofilms. *B. burgdorferi*, however, utilizes manganese for biological processes instead of iron, which means that lactoferrin antibacterial effects on *B. burgdorferi* biofilm must have a different mechanism which needs to be further investigated. [10] Manganese may act in the same way as iron acts for *P. aeruginosa*. Clinical studies with lactoferrin show no significant side effects and is a readily found protein in cow and human milk.

Further research will be performed for my Honors Thesis where I plan to utilize additional tests including Live/Dead Assay for logarithmic and stationary growth phase combined with Live/Dead microscope direct counting method, Total Carbohydrate assay as well as any additional tests that have their technique finalized within UNH's Lyme Disease Lab over the following months. These tests will demonstrate effects of lactoferrin on the different components of *B. burgdorferi* biofilm, such as the protective mucopolysaccharide and extracellular DNA layers to better understand the mechanism of lactoferrin. Future results from this research may provide promising data for a safe, affordable, and highly effective approach to eliminate *B. burgdorferi* infections in Lyme disease patients.

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Biography:

Dylan Haenel is a senior at the University of New Haven majoring in Biotechnology. He works as an Academic Peer Mentor for Bethel Hall at the university. He also tutors biology at the Center for Learning Resources. He plans to participate in international internships to gain experience and ultimately wants to pursue a Ph.D. in a branch of biology