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The antimicrobial effects of propolis and bee venom on *Spirochaeta cytophaga* cultures as a model for Lyme disease treatment

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Abstract
Lyme disease, caused by tick borne infections of the bacteria *Borrelia burgdorferi*, is typically treated with antibiotics. However, because not all patients respond to this treatment and excessive antibiotic use can lead to resistance making future infections difficult to treat, alternative treatments are needed. Bee venom and propolis are bee secretions that both display antimicrobial properties and therefore have potential to treat bacterial infections. In order to safely determine the antimicrobial effects of propolis and bee venom on harmful bacteria without the risks of exposure to the pathogenic bacteria, a bacteria species with a homologous outer membrane (*Spirochaeta cytophaga*) to the Lyme disease causing bacteria was used as a model in this research. To test the antimicrobial effects of propolis and bee venom, *S. cytophaga* cultures, treated with varying treatment types, were quantified daily using optical density spectrophotometry to approximate the time interval required for treatment to become effective. The efficacy of treatment was determined through observation of a statistically significant decrease in the optical density of cultures. Dark field microscopy was subsequently utilized to confirm that the outer membrane was the structure acted upon by the experimental treatments. Finally, the minimal inhibitory concentration for bee venom, propolis, and both combined was calculated using a microplate reader. The results indicate that the combined propolis and bee venom treatment was significantly more effective than other treatments. This combined treatment had a minimal inhibitory concentration of 2.5% propolis and 83.75 micrograms per milliliter bee venom. Additionally, both propolis and bee venom acted on the outer membrane of *S. cytophaga* which suggests that the effects of these treatments are potentially similar on the pathogenic bacteria.

Keywords: propolis, bee venom, antimicrobial, treatment, lyme disease

1. Introduction
Lyme disease is a tick borne infection of *Borrelia burgdorferi* that is highly pervasive with over 300,000 cases nationally according to the Center for Disease Control and Disease Prevention Lyme disease data and statistics (CDC, 2017). Lyme disease is typically treated with antibiotics, but alternatives to antibiotics are needed as the excessive use of antibiotics promotes the growth of resistant bacteria (Socarras *et al.*, 2017; Terekhoval *et al.*, 2002; Lubke *et al.*, 1997) [13, 8]. The threat is seen in laboratory testing of strains of *B. burgdorferi* where the bacteria displayed an increasing resistance to antibiotics such as Erythromycin (Terekhoval *et al.*, 2002). Furthermore, there are cases of Lyme disease that are exacerbated by the excessive use of antibiotics contributing to heightened fatigue, musculoskeletal aches, and lapses in cognitive function (Auwaerter, 2007) [1]. This is commonly referred to as "chronic Lyme disease." In order to address antibiotic resistance in Lyme disease, there is an interest in researching alternatives (Socarras *et al.*, 2017; Lubke *et al.*, 1997) [13, 8]. Bee products, such as propolis and bee venom, may have potential to act as alternatives to pharmaceutical antibiotics as many of these products have already displayed antimicrobial properties (Cornara *et al.*, 2017) [2].

Bee venom has the ability to kill bacteria because of its main component: melittin (Socarras *et al.*, 2017) [13]. An experiment testing melittin as an antimicrobial agent against *B. burgdorferi* growth measured the optical density (OD) of bacterial cultures with a spectrophotometer daily over the course of one week (Lubke *et al.*, 1997) [8]. This study found that melittin was successful in reducing the quantity of live bacteria after the third day post treatment. This research also identified the outer membrane of the bacterium as the structure that is affected by the treatment through observation of the outer membrane lysis and blebbing as a result of exposure to melittin. Although melittin has already shown antimicrobial effects when treating Lyme disease, it is a limited resource and not entirely effective on its own (Lubke *et al.*, 1997) [8].

In order to supplement mellitin's efficacy, additional alternative antimicrobial agents should be explored. Propolis is a resin secretion also produced by bees that has displayed antimicrobial properties in past studies (Coutinho, 2012; Grange, 1990) [3, 4]. Unlike melittin, propolis has yet to be tested as an antibacterial treatment for Lyme disease and is more accessible than bee venom. In a 2012 experiment, propolis was tested as a potential treatment for the oral bacterial infection known as periodontitis, and the researchers found that propolis was effective against periodontal bacteria (Coutinho, 2012) [3]. This research used a 20% propolis solution to test its antimicrobial properties. Another study using propolis on a diverse range of bacteria found that

propolis consistently displayed the ability to combat varied infections (Grange, 1990) [4]. In this study, the antibacterial properties of propolis were tested on 21 different strains of bacteria such as *Escherichia coli*, and researchers concluded that propolis was successful in decreasing the growth of every single strain. Because propolis is accessible and has demonstrated antimicrobial properties, it has potential to be an effective alternative treatment for Lyme disease. This research aims to address the use of propolis alone and in combination with bee venom as alternative treatments for Lyme disease.

In order to maintain safety in a biological safety level 1 lab, a nonpathogenic model organism for *B. burgdorferi* should be used to test efficacy while minimizing potential health hazards. Of all genera in the class Spirochete, *Borrelia* and *Spirochaet*a are the most morphologically similar (Remington, 2011) [12]. Additionally, within the Spirochete class, *Spirochaeta* and *Borrelia* are the most genetically similar genera (Paster *et al.*, 1991) [11]. Furthermore, *Spirochaetes* and *Borrelias* have the same high signal-channel conductance in their outer membranes as well as main outer membrane proteins and porins (Thein, 2009) [16]. These similarities in outer membrane morphology and composition are important because they provide evidence that *S. cytophaga* has potential to be an effective nonpathogenic model for *B. burgdorferi* in testing of antimicrobial agents. The outer membrane of the treated *S. cytophaga* must be observed because the outer membrane is the affected structure of *B. burgdorferi* when treated with antimicrobial substances, and it is also the homologous structure between *S. cytophaga* and *B. burgdorferi*. When treated with melittin, the outer membrane of *B. burgdorferi* displays lysis, breaks in the outer membrane, and blebbing, bulges of the outer membrane, which causes the death of the bacteria (Lubke *et al.*, 1997) [8]. If the treatments of interest do not affect *S. cytophaga's* outer membrane, the results may not be applicable to Lyme disease. In order to confirm lysis and blebbing on a Spirochete, a dark field microscope set at 400X magnification can be used (Listgarten *et al.*, 2005) [7].

This research investigated whether propolis alone and in combination with bee venom effectively inhibits growth of *S. cytophaga.* The optical density of bacteria treated with propolis, bee venom, and both combined was measured daily for five days. This displayed the duration of time necessary for treatments to cause a decrease in live bacteria in treated broth suspensions. After measurement of optical density, treated bacteria was observed under a dark field microscope to confirm that the outer membrane was the affected structure. Finally, the minimal inhibitory concentration of treatments (propolis, bee venom, and the treatments combined) was determined with a microplate reader. It was hypothesized that the most effective treatment would be the use of bee venom and propolis together at the highest concentration. The results of this research may contribute to the development of a new treatment for Lyme disease: a highly pervasive disease with over 300,000 cases in the US annually with upwards of 20% of diagnoses unsuccessfully treated by traditional antibiotics (CDC, 2017).

2. Materials and Methods

2.1 Rehydrating *S. cytophaga* Culture

The *S. cytophaga* strain ATCC 10010™ was obtained from ATCC and rehydrated according to the manufacturer's recommended protocol (Medium 329: Mineral salts broth, ATCC) in a Class II Type A2 biological safety cabinet.

2.2 Optical Density Spectrophotometry

16 cuvettes of growth medium and treatment were prepared according to the treatments described in Table 1. Note that the "pure growth medium" described in Table 1 refers to the Medium 329: Mineral salts broth described previously. Additionally, the bee venom used throughout research was medical grade and therefore contained 60% melitin. The optical densities of the samples were recorded directly after their preparation in order to ensure equal bacterial distribution, and this data was recorded as "Day 1." The change in bacterial density was measured daily with a Vernier Spectro Vis Plus spectrophotometer set to a wavelength of 600 nm, for 5 days in order to quantify the time required for each treatment to reduce the optical density of the bacterial suspension. The raw data was analyzed with 95% confidence intervals to evaluate significance as has been previously used to determine significance in similar studies (Hall *et al.*, 2013) [5]. The optical density values were interpreted to determine the length of time required to significantly reduce live bacterial colonies.

Table 1: Description of ODS cuvette set-up that outlines the trials for each treatment, contents of all 16 cuvettes, and support for determined treatments.

Treatment Type	Number of Trials	Volume of Pure Growth Medium	Quantity of Treatment	Concentration of Treatment	Total Volume in Cuvette
Control	4	2.00 mL	No Treatment	No Treatment	2.00 mL
Bee Venom	4	2.00 mL	1340 µg Bee Venom	670 µg/mL Bee Venom containing 400 µg/mL Melittin (Lubke *et al.*, 1997) [8]	2.00 mL
Propolis	4	1.60 mL	0.40 mL Propolis	20% propolis (Coutinho, 2012) [3]	2.00 mL
Propolis + Bee Venom	4	1.60 mL	0.40 mL Propolis + 1340 µg Bee Venom	20% propolis + 670 µg/mL Bee Venom	2.00 mL

2.3 Dark Field Microscopy

Dark field microscopy can be used in order to view Spirochetes with magnification, and normal light microscopes can be converted into dark field microscopes by attaching an opaque object to the condenser to scatter the central light so that only oblique rays hit the specimen (Listgarten et al., 2005) [7]. A binocular compound light microscope (National Optical, Texas) was converted into a dark field microscope by using an appropriate dark field condenser (National Optical 927 Darkfield Microscope Attachment, Texas). Bacteria samples were obtained and observed on day five of the ODS procedure with slides sampled from each treatment type

(Control, Propolis, Bee Venom, and Propolis + Bee Venom). The Spirochetes' outer membranes were compared to confirm that the treatments killed the *S. cytophaga* through outer membrane lysis as previous research has displayed this to be the method of in which melittin kills *B. burgdorferi* (Lubke *et al*., 1997) [8].

2.4 Minimal Inhibitory Concentration

The minimal inhibitory concentration (MIC) of antimicrobial treatments is a useful measurement to determine the minimum dosage of an antimicrobial treatment that maintains efficacy and can be determined using a plate reader (Nguyen *et al.*, 2018; Weigand *et al*., 2008) [10]. The bacteria culture was diluted to the standard turbidity (10^8 cfu/mL) that is necessary in order to perform a minimal inhibitory concentration test (Weigand *et al*., 2008). Four treatment types (Control, Bee Venom, Propolis, and Propolis + Bee Venom), seven concentrations, and three trials were tested in order to determine the minimal inhibitory concentration of each treatment. The wells were filled with varying concentrations of the previously indicated ODS treatments (Table 1). Serial dilutions of MIC solutions were created starting at 200% of the ODS treatments and were reduced by a factor of two for each group until reaching 3.13% as indicated in Table 2. The plate was incubated at 26º C for 96 hours and read to determine optical density using an iMark microplate reader (Bio Rad, California) with a wavelength of 600 nanometers. The average and the 95% confidence interval were determined for each group as similar studies have used to determine significance (Nguyen *et al.*, 2018) [10]. The optical density values were interpreted to find the minimal concentration of treatment that inhibited the bacteria with the same statistical strength as the highest concentration of treatment.

3. Results

3.1 Optical density spectrophotometry

The initial average optical absorbance values for all four groups were between 0.745 and 0.755 absorbance at a wavelength of 600 nanometers (Fig. 1). Throughout the 5 day trial, the control samples average absorbance increased slightly and plateaued. The bee venom treatment average optical density increased over the first three days of the trial then decreased during days four and five. The propolis and combined treatment averages both decreased at the same rate over the first four days of the trial; however, on the fifth day, the combined treatment average was significantly lower than the propolis treatment average. The combined treatment average absorbance remained significantly lower than the bee venom throughout the trial and the difference was statistically significant on all days except the fifth. After five days of exposure, the propolis, bee venom, and combined treatment averages had significantly lower optical density values than the control.

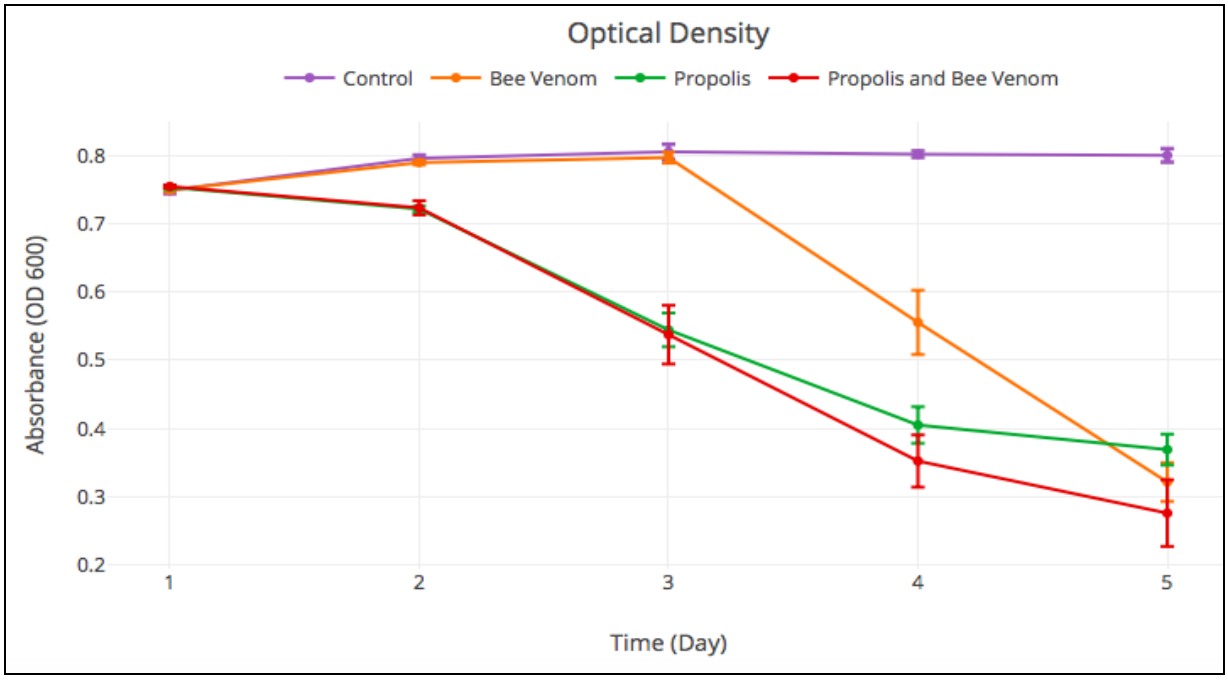

Fig 1: OD of control, venom, propolis, and combined treatment over five days measured with a wavelength of 600 nm. Four trials per group were performed with their averages plotted with a 95% confidence interval of error.

3.2 Dark Field Microscopy

The observed *S. cytophaga* sample from the control treatment possessed an outer membrane that appeared completely in tact with no lysing or blebbing (Fig. 2A). The *S. cytophaga* samples from the experimental groups all displayed various degrees of lysis and blebbing with a complete fracture across the bacteria treated with propolis (Fig. 2). The bacteria treated with solely bee venom (Fig. 2B) only displayed blebbing and lysis whereas the bacteria treated with propolis (Fig. 2C) and both propolis and bee venom (Fig. 2D) displayed lysis, blebbing, and fractured outer membranes compared to the control and typical Spirochete.

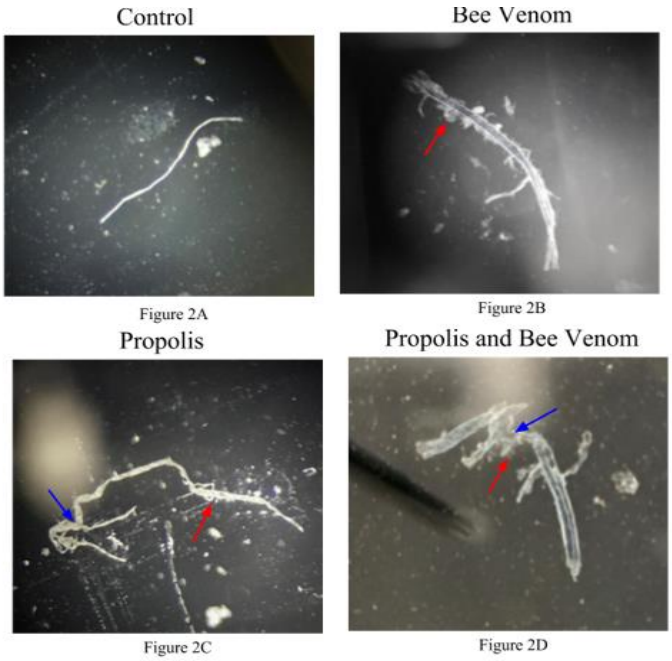

Fig 2: Images of *S. cytophaga* at 400X magnification observed after five days of exposure to specified treated growth media. There is a presence of lysis and blebbing in all groups except the control (indicated with red arrows), and there is a presence of outer membrane fracture in groups with propolis (indicated with blue arrows).

3.3 Minimal Inhibitory Concentration

At 3.125% concentration of all treatments, there was no significant difference between the quantity of bacteria with the combined propolis and bee venom and the control, but all other percent concentrations of combined treatment displayed significantly less bacteria than the control (Table 2; Fig. 3). At 12.5% concentration, the optical density of the combined treatment was not significantly different than the combined treatment at 200%. Additionally, at 12.5% concentration, the optical density of the combined treatment was significantly lower than the individual propolis and bee venom groups.

Table 2: Raw data of MIC absorbance value averages (with 95% confidence interval error values). The percent concentrations refer to the percentage of ODS treatment concentrations used (100% = 20% propolis + 670 µg/mL Bee Venom).

Treatment Type	Percent Concentration of ODS Procedure Treatment (p<0.05 error)						
	200%	100%	50%	25%	12.50%	7.25%	3.13%
Propolis	0.105 (0.016)	0.165 (0.009)	0.198 (0.014)	0.208 (0.009)	0.238 (0.013)	0.557 (0.237)	0.849 (0.050)
Bee Venom	0.0577 (0.021)	0.108 (0.027)	0.104 (0.097)	0.151 (0.104)	0.426 (0.162)	0.560 (0.215)	0.743 (0.255)
Combined	0.054 (0.009)	0.057 (0.006)	0.082 (0.027)	0.143 (0.074)	0.154 (0.083)	0.369 (0.072)	0.720 (0.340)
Control	0.851 (0.018)						

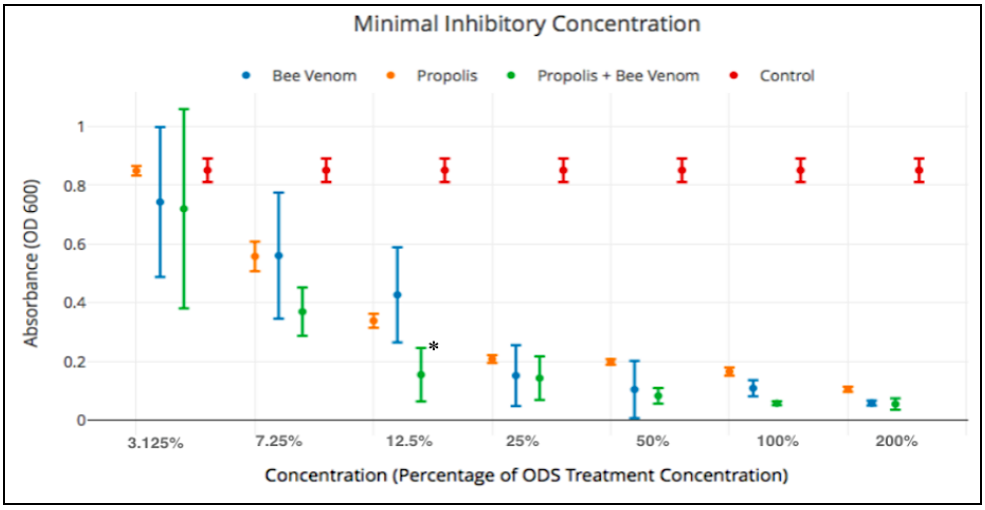

Fig 3: Minimal Inhibitory Concentration, of tested percentages of the OD procedure's treatment measured with a wavelength of 600 nm using a

Bio Rad iMark microplate reader.

The averages of three trials were plotted with a 95% confidence interval as error.

4. Discussion

The Optical Density Spectrophotometry procedure displayed that the combined treatment was significantly more successful in eliminating bacteria than the bee venom on days two through four and significantly more successful in eliminating bacteria on day five than propolis. The bacteria in the control group quickly reached its carrying capacity, and after day three, there was a significant distinction between all treated groups and the control which indicated the treatments' potential success in inhibiting the growth of *S. cytophaga*. The bee venom treatment took slightly more than three days to become active which is consistent with previous findings (Lubke *et al.*, 1997) [8]. Contrastingly, the propolis decreased the optical density of the bacteria immediately which indicates that propolis' antimicrobial properties are fast-acting when treating *S. cytophaga*. The combined treatment group displayed similar trends to both of its isolated components as it is fast-acting, similar to the propolis, and causes a significant decline in live bacteria between days three and four, similar to the bee venom. The combined treatment group was more effective in treating the bacteria than the bee venom alone which supports the conclusion that bee venom supplemented with propolis could be an effective antibacterial against *S. cytophaga*. Additionally, it is likely that this antibacterial would require at least three days for both the propolis and bee venom to be effective.

The dark field observation of treated *S. cytophaga* confirmed that the affected structure was the outer membrane of the Spirochete. Additionally, previous research has found that the ruptures of outer membrane of *B. burgdorferi* is the mechanism in which melittin treats Lyme disease (Lubke *et al.*, 1997) [8]. In this research, the propolis caused a warped shape in *S. cytophaga* along with blebbing and lysis, and the bee venom caused solely lysis and blebbing which was consistent with previous findings of melittin treated *B. burgdorferi* (Lubke *et al.*, 1997) [8]. The outer membrane is the homologous structure between *B. burgdorferi* and *S. cytophaga*, and therefore it is possible that the impact of the tested treatments on *S. cytophaga* could be a successful antibacterial against *B. burgdorferi* as well.

The minimal inhibitory concentration for the combined treatment was 12.5% as it was the lowest concentration with an optical density that did not differ significantly from its higher treatment concentration absorbance values. The minimal inhibitory concentrations for the bee venom and propolis, as separate treatments, was 25% because there was no significant difference between the groups' optical densities at 25% and their higher treatment concentration absorbance values. This data indicates that the 12.5% combined treatment is the minimum concentration and treatment type that exhibits maximum efficacy. This percentage refers to the quantity of propolis and bee venom used in the optical density spectrophotometry procedure where 20% propolis and 670 micrograms per milliliter of bee venom venom were used. 12.5% of this combined treatment amounts to 2.5% propolis with 83.75 micrograms per milliliter of bee venom. Combined, these are the minimal inhibitory concentrations for antibacterial treatment of *S. cytophaga* cultures.

Potential limitations of this research include the small sample sizes contributing to larger error amongst recorded data. For example, the combined treatment group at 3.125% concentration, in the MIC procedure, had a large range of error. Had a larger sample size been tested, the averages may have been more precise and could have influenced the conclusions of minimal inhibitory concentration. If repeated, this research should include more samples to allow for greater resolution between tested groups. Although this research predicts the combined treatment's antibacterial efficacy against *B. burgdorferi*, subsequent testing on the Lyme disease causing bacteria is necessary to confirm this hypothesis. Additionally, this research was performed in vitro, and therefore testing the combined treatment in vivo is necessary to determine the treatment's effects within the greater biological system of the pathogen's host.

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6. References

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