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Evaluating Anecdotal Lyme Disease Treatments for Borrelia-inhibitory Properties

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Evaluating Anecdotal Lyme Disease Treatments for Borrelia-inhibitory Properties

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Honors Thesis, 2018/9

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Wellesley College
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And to my parents and friends who have helped so much, in so many emotional and tangible ways, but most of all to my children who paid so high a price for my education. I love you, I’m sorry.
Abstract

Lyme disease is the fastest growing vector-borne illness in the United States. Its causative factors, are the spirochetal bacterium *Borrelia burgdorferi* and its close relatives. Infection in humans hard to treat, and long term, or chronic, illness follows initial *Borrelia* infection approximately 20% of the time. Many sufferers turn to non-traditional methods of treatment. This study sought to identify and test natural products for anti-borrelian qualities, using anecdotal success as an initial filter. Thirty-five herbal Lyme disease treatments were so identified. Extracts from these materials were made using three solvents, and tested against *Borrelia burgdorferi*. Anecdotal success proved to be an effective filter for *Borrelia* inhibition, allowing us to identify several extracts which inhibited its growth, and also several growth enhancers, each of which should be further investigated as relevant to understanding and treating *Borrelia* infections in humans.
**Abbreviations and Definitions**

ATP- adenosine triphosphate- energy currency of the cell  
BbGFP-test strain of *Borrelia burgdorferi* with GFP marker  
CDC- center for disease control  
EM- erythema migrans, the representative bulls-eye rash seen in 40% of *Borrelia* infections  
EPS- extracellular polymeric substances (goo around biofilm)  
FlaB- core flagellar protein in Borrelia  
GFP- green fluorescent protein, a marker added via kanamycin-retained plasmid to allow detection via fluorescence readings  
GlcNAc - sugar, N-Acetyl Glucosamine  
IAFGP- ixodes antifreeze glycoprotein  
LPS-Liposaccharide component of some bacterial cell membranes  
MIC- minimum inhibitory concentration  
Negative control- area demonstrating unaltered natural growth of bacteria, for comparison  
OspA, Osp C- outer surface protein A, C, etc.  
PICC- peripherally inserted central catheter- line inserted in the brachial artery (upper arm) that extends to the heart and administers intravenous pharmaceuticals directly, for most effective full-body distribution.  
Positive control- area demonstrating killed or inhibited state using known compound, for comparison  
PTLDS-post treatment Lyme disease syndrome  
SOD- super oxide dismutase
Background & Significance

HISTORY AND DISTRIBUTION OF LYME DISEASE

Lyme disease is the fastest growing vector-borne illness in the temperate world, (figure 1a) with 822 new cases every day and 300,000 new infections documented yearly in the U.S. alone (figure 1b) (CDC). In the United States, Lyme disease was ranked among the six most common Nationally Notifiable diseases by the Centers for Disease Control in 2015. The geographic concentration of infection is notable, with 95% of new diagnoses originating from only 14 states (Connecticut, Delaware, Maine, Maryland, Massachusetts, Minnesota, New Hampshire, New Jersey, New York, Pennsylvania, Rhode Island, Vermont, Virginia and Wisconsin) (CDC) (figure 1b).

![Figure 1: World and U.S. distribution of Lyme disease. a. documented Lyme disease in the temperate world (red). b. 95% of new Lyme disease cases in U.S. are in just 14 states. Each dot represents a county with Lyme disease diagnosis. Figure adapted from Center For Disease Control.](image)

Previously known as the summer flu for its initial flu-like symptoms, Lyme disease got its name from the Connecticut town where its causative factor, the spirochetal bacterium *Borrelia burgdorferi*, was first isolated by Dr. William Burgdorfer in 1982 (Steere et al., 1983). Despite recent attention stemming from pronounced increase in documented cases, the pathogen is neither new to humans, nor an American phenomenon. The earliest evidence of *Borrelia* infection comes from Ötzi, a 5300-year old ice mummy uncovered on the Tisenjoch Pass of the Italian Alps in 1991. Ötzi’s genome was sequenced in 2012, and among his cells, 60% of *Borrelia*’s genome was also found (Keller et al., 2012). It was the only pathogenic bacterium isolated from the mummy.
ECOLOGICAL PLAYERS

The geographical density and rampant spread of the disease becomes more understandable with knowledge of *B. burgdorferi*’s ecological niche. This etiologic factor is a fastidious, microaerophilic obligate parasite which has evolved a multi-host lifecycle of three main components: the bacterium, its vector the *ixodes* tick, and the biological reservoir, white footed mice (Radolf et al., 2012). There are additionally two notable ancillary participants: a vector maintenance species (deer) and accidental hosts (notably, humans and canines).

Because *Borrelia* is not able to survive naturally outside of a biological host, and is not transmitted via either mammalian or arachnid ova (Breuner et al., 2018), its population must continually be maintained across the arachnid and vertebrate hosts by blood transmission. Relying on differential gene expression to exist in the disparate physiological conditions of these animals, *Borrelia* travels via the bite of its primary vector, the hard-bodied *ixodes* tick. These arachnids require a blood meal to molt or lay eggs, and it is through these blood meals that both the initial arachnid infection and later transmission to mammalian hosts are accomplished (figure 2a).

*Borrelia*’s absolute dependence on tick as vector means that its infection rates are tied to the life cycle of the vector species such that the acute Lyme disease infection rates correlate to both the environmental presence of nymphal ticks (figure 2b), and to the size and distribution of the infected tick
population as a whole. The warmer, wetter, environmental conditions afforded by climate change allow expansion of arthropod-friendly habitats (Ostfeld and Brunner, 2015), with the Center for Disease Control and Prevention estimating that at current rates of climate change, relevant tick habitats will increase 213% by the year 2080 (CDC). Further, as human civilization expands, overlap between our habitats and those of *Borrelia*’s biological vector leads to higher rates of human exposure and infection through proximity.

Another species relevant to *Borrelia*’s life-cycle is the white footed mouse (*Peromyscus leucopus*). As primary biological reservoir for the bacterium, the health and size of this small rodent’s population heavily impacts Lyme disease infection rates, demonstrating increase of new infections, for example, during years with an abundance of acorns, a primary calorie source of the reservoir species. Mice pose an especially significant problem for *Borrelia* control due to adaptive factors which make them an ideal reservoir.

The white footed mouse displays very little immune response to the bacterium (Ullmann et al., 2003) (figure 3a), and therefore does not suffer from movement-inhibiting, pain-inducing inflammatory symptoms present in most other hosts. Similar species, such as chipmunks and rabbits, transmit *Borrelia* at 50% the rate of white footed mice (figure 3b) within the same Lyme disease endemic habitats, despite similar phylogeny and food sources due to greater immune response. Because of their lack of humoral bacteriolysis, mice have a comparatively higher systemic infection level without significantly decreased locomotion or neurological involvement, making them ideal
biological reservoirs. In contrast, the bacterial dose required for severe symptomatology in humans is very low, at one bacterium per 10mL (Wolgemuth, 2016) with symptom-decreased mobility at a low bacterial load they offer no evolutionary benefit to the bacterium and are therefore characterized as incidental hosts.

Second, white footed mice derive specific benefit from their association with ticks: frostbite resistance. Feeding ticks contribute small amounts of ixodes antifreeze glycoprotein (IAFGP) to their host (Heisig et al., 2015; Neelakanta et al., 2010). In sufficient concentration, accomplished with relative ease on small bodies with many ticks, this glycerol imparts protection to the white footed mice expanding its natural habitat, and making ticks desirable habitat partners. Nesting mice, in return, provide a target-rich environment of hosts for newly-hatched ticks.

This tripartite symbiosis between bacterium, biological vector and reservoir species is a primary factor in the spread of Lyme disease, but there are two relevant ancillary participants as well: deer and incidental hosts such as humans and canines. Deer are an essential part of the vector life-cycle, as the primary hosts for ixodes pre-ovulation blood meals (CDC)(figure 3a), but they are not carriers of *Borrelia*, demonstrating near one hundred percent immune kill rate of the bacterium in laboratory tests (figure 3c) (Ullmann et al., 2003). In the US distribution map (figure 1b), spread of *Borrelia* is slow- moving, perhaps, at the speed of mouse migration, instead of that of deer. Transmission by deer would display wide, faster coverage, due to their broad and overlapping migratory routes. Instead, deer support and transport the tick adults and are most often the source of the blood meal necessary to form eggs, but ticks feeding on deer become *Borrelia* free (Roome et al., 2017). This suggests that it may be evolutionarily beneficial to remove *Borrelia* as caloric competitors for egg production resources. Ticks return to mice and small rodents for the laying of eggs, and the larval ticks become infected with *Borrelia* from these mouse reservoirs.

**BORRELIA: BEHAVIOR, STRUCTURE AND EXPRESSION**

*Borrelia burgdorferi* is one of many related spirochetal bacteria (Margos et al., 2018) with a multiple-host life cycle (figure 3a), truncated metabolism and plasmid-modified genetic profile (Casjens et al., 2017) comprised of a small linear chromosome containing 853 genes, and 17-22 relatively large linear and circular plasmids that contain additional information essential for virulence and infectivity (Kenedy et
It is virulent and highly invasive (Aslam et al., 2017), and notoriously difficult to work with under lab conditions. Its fastidiousness, slow-growth, microaerophilic requirements, and genetic intractability, represent mechanisms unique in the known world (Troxell et al., 2012), and there is a growing library of research devoted to deciphering its secrets.

**Membrane strategies**

Categorized as gram-negative-similar, due to its diderm structure but lack of gram-negative-characteristic external liposaccharide proteins, *Borrelia’s* external membrane is resistant to polar compound intrusion, helping it to resist many antibiotic compounds ( Scorciapino et al., 2017). The membrane also provides more specific immune protection imparted by its multi-host adaptation, in the form of differential expression, antigen switching, and hiding of intrinsic proteins.

Immune response often relies on recognition of external proteins, and therefore control of protein exposure is a potent immune-evasive adaptation. In *Borrelia*, protein expression is controlled by the Rrp2-RpoN-RpoS regulatory pathway, and triggered by environment, specifically the *ixodes* blood meal ( Groshong and Blevins, 2014). Outer surface protein C (OspC) is a primary indicator of mammalian *Borrelia* infection, while Outer surface protein A (OspA) is expressed primarily in arachnid hosts (Kenedy et al., 2012). As central regulator of this pathway, and therefore of differential protein expression necessary for transfer and survival between arthropod and mammalian hosts, RpoS mediates the OspA/OspC balance to optimize infection, dissemination and bacterial survival (Yang et al., 2003).

Because these externally expressed proteins are strongly immunogenic, provoking a hearty immune response at very low concentration once recognized
by the body, *Borrelia burgdorferi* has evolved an elegant immune evasive solution. The external membrane varies presentation of immune-exposed proteins to reduce antibody formation. This antigen switching, moves specific surface-exposed lipoproteins-targeted to the external leaflet by default after translation- to a secondary location within the bacterial periplasm (Hyde, 2017). The behavior relocation is based on sequence-specific signals (Schulze and Zuckert, 2006) and renders the proteins invisible to the immune system. Additionally, several plasmids have been found to be responsible for outer surface protein production, presenting a huge, and ever-new array of membrane proteins to foil immune recognition (Cullen et al., 2004). The variety and rotation of membrane-presented proteins makes antigen testing for Lyme disease diagnosis problematic.

Another way in which *Borrelia* uses its outer membrane to guard against immune response is purely structural: it expresses certain essential proteins (notably the flagella) only within the periplasmic space (figure 5). As opposed to the external flagella present in most known prokaryotes, *B. Burgdorferi* has 7-12 periplasmic flagella between the cell body and outer membrane which confer both its distinctive spiral shape and eukaryote-similar flat-wave movement patterns (Motaleb et al., 2000). Mutants deficient in Fla-B (a structural flagellar protein) are both non-motile and non-spiral, confirming this relationship (Wolgemuth et al., 2006).

**Nutrient use:**

During infection and dissemination in a mammalian host, flagella impart both directional control, and velocity: allowing the spirochete to exceed the speed of neutrophils, the fastest human immune cell, by 2 orders of magnitude and making them an important, and effective, part of the bacterium’s infective cycle.
However, this speed has a cost, and flagellar motors are nutrient-greedy, high consumers of ATP. To feed this need, the fastidious *Borrelia* has only seven carbon sources: Glucose, GlcNAc, glycerol, chitobiose, trehalose, mannose and maltose, and shows preferential use of glucose due to several transport shortcuts which make it the least resource-intensive to metabolize (Troy et al., 2016; Vechtova et al., 2018).

The bacterium travels optimally in the structure and resistance of extra-cellular matrix material which informs laboratory use of semisolid media during cultivation (Harman et al., 2012). N-Acetyl Glucosamine (GlcNAc), which comprises the majority of this matrix in mammals, is not only one of the seven carbon sources used by *Borrelia* for metabolic purposes, it also acts as a chemoattractant (Bakker et al., 2007) and may be a factor in sequestration in GlcNAc-rich cartilaginous sites.

*Borrelia* lacks coding for the enzymes necessary for full aerobic metabolism and does not possess the ability to perform a TCA cycle, oxidative phosphorylation, or electron transport (Corona and Schwartz, 2015). It relies solely on glycolysis and abundant host resources for ATP production, and has limited biosynthetic capability, with no complete pathways for *de novo* synthesis of fatty acids, amino acids, nucleotides, or co-factors, and relying instead on multiple and redundant transport proteins for various nutrients and cofactors, making it an obligate parasite (von Lackum and Stevenson, 2005).

The microbe’s host-specific behaviors are controlled by the presence of host-specific nutrients and environmental conditions which in turn govern gene expression, viability, and movement. The bacterium’s complicated life cycle and condensed genome force it to rely on different carbon sources in each host, and it exhibits host-specific morphologies and protein expression seemingly tied to this reliance (Steere et al., 1983).

The bacterium’s paucity of metabolic machinery shows in several essential ways: first, in speed of growth. Due to its sole reliance on glycolysis for ATP (Corona and Schwartz, 2015; von Lackum and Stevenson, 2005). *Borrelia* grows very slowly- with a generation of 12-24 hours. This makes cultivation and treatment challenging for both temporal reasons, as duration of single test growth is 2 weeks, and also for
reasons of contamination, because short-generation contaminants, if present, will out-grow and out-compete *Borrelia* in the very rich media it requires.

Metabolic limitations are also displayed in behavior: two of its seven carbon sources (glucose and GlcNAc) are strong chemoattractants, inducing migration; and a preponderance of a third (glycerol) triggers a semi-dormant state of low movement and reproduction. This stillness is exhibited in the hemolymph, or gut, of an unfed arachnid seemingly because 3-carbon glycerol, the most abundant nutrient in that habitat, does not provide enough ATP for the energy-expensive flagellar motors to be fully active. The unfed tick midgut is oxygen-poor, cold and alkaline. This condition persists until one of three post-infective bloodmeals is taken by the arachnid host. Once a blood meal is taken, the increase in nutrients, temperature, acidity, cell density and oxygenation activate chemotaxis and differential expression of external proteins (Caimano et al., 2016). These environmental changes trigger the primary regulatory pathway (Rrp2-RpoN-RpoS), which signals outer surface protein activation/repression (Sapi et al., 2016) and other differentially expressed proteins allow binding of plasminogen activators present in the mammalian blood, and production of proteolytic plasmin which allows migration through the tick endothelium to the salivary glands for injection into the mammal host during feeding (Carroll et al., 1999; Coleman et al., 1997) change and transit takes some hours after exposure to mammalian blood, after which point *Borrelia* may be transmitted to a mammalian host. After 6 hours 4%, 12 hours 10% and after 4 days of attachment there is 100% probability of *Borrelia* transmission from an infected tick (CDC).

In the salivary glands, *Borrelia* binds immunosuppressant salp15 protein found in tick saliva, to inhibit T-cell activation in mammals and facilitate early migration, and in the nutrient-rich mammalian host, it again expresses lytic complement complexes which aid in immune evasion by extracellular matrix degradation (Kurtenbach et al., 2002) during flagellar travel.

Once in the mammal, spirochetes are able to disseminate quickly, moving to immune-deficient compartments such as joints before immune cells can capture them, (Malawista and de Boisfleury Chevance, 2008) and, in some cases, an autoimmune effect occurs, and the patient’s own immune system joins *Borrelia’s* degradation of the extra-cellular matrix, destroying tissue.
DISEASE AND TREATMENT

*Borrelia burgdorferi* infection in humans is responsible for an array of symptoms from fever to palsy, including paralysis, cognitive impairment, and unremitting pain. Due to the constellation of symptoms, Lyme disease is often misdiagnosed as fibromyalgia, lupus, stroke, depression, schizophrenia, and any number of minor ailments (Berndtson, 2013). This shared symptomology has prompted medical professionals to dub Lyme disease “The great imitator” (a nickname previously used for syphilis- also caused by a spirochete). Combined with the low bacterial load necessary for significant illness and poor antibody testing, this confusion has proven an impediment to timely treatment.

Diagnosis of borreliosis based on either symptoms or serology has proven difficult. Clinical diagnosis of Lyme disease is based on four primary acute infection symptoms. 1. Erythema migrans, or bullseye rash (Chaaya et al., 2016) which is exhibited by less than 40% of infected humans and indicates cutaneous dissemination. 2. knee effusion- a symptom of immune response to hiding spirochetes in the cartilaginous knee which indicates humoral dissemination 3. Bell’s palsy -paralyzation of half of the face which indicates neurological dissemination or 4. or a positive antibody test demonstrating an immune response to 3 or more specific *Borrelia* proteins, whose problematic nature we have already discussed (Dessau et al., 2018).

Even after diagnosis, treatment of the disease is not straightforward. Oral doxycycline, a protein synthesis inhibitor which is *Borrelia*-static, rather than *borreliacidal*, can be effective very early in the acute infection stage (previous to sequestration or neurological involvement), but disseminated *Borrelia* cannot be cleared in this way. The spirochete’s sequestration behavior requires widespread minimum inhibitory concentration (MIC) of effective anti-*Borrelia* antibiotics in all compartments (intra as well as inter cellular, vascular as well as a-vascular, and both sides of the blood/brain barrier in cases with neurological involvement). This is difficult because dosing required to reach intracellular MIC of pharmaceuticals can result in toxic serum levels. Additionally, *Borrelia*s lengthy12-24 hour generation time complicates treatments that rely on drug density during reproduction, as it is difficult to maintain sufficient and constant blood and tissue concentration for the multiple generation periods necessary to address an active infection.
And finally, although *Borrelia* can (and does) cross the blood/brain barrier to provoke severe neurological symptoms (Ramesh et al., 2003) not all commonly used pharmaceutical can- which limits drug choice.

Even in the face of what is thought to be appropriate antibiotic treatment, around 20% of cases still experience disease progression and long-term illness (CDC). Continued illness seems to depend to some extent on individual immunity, and some early infections don’t show the specific hallmark symptoms associated with Lyme disease, instead presenting as flu-like symptoms, muscle cramps or migraine headaches and remaining untreated until more serious signs emerge. However, later illness is often much more pronounced and characterized by ongoing exhaustion, pain, cognitive impairment, autoimmune symptoms, metabolic disruption, psychological symptoms, (Ramesh et al., 2003)and later, related, diagnoses including lupus, sarcoidosis, Alzheimer’s disease, multiple sclerosis, non-Hodgkin’s lymphoma and glioblastoma.(Schollkopf et al., 2008)

One school of thought holds that continued or relapsing illness after *Borrelia* infection is due to insufficient eradication of the spirochete. This is often referred to as Chronic Lyme disease, because it is believed to result from a remaining pathogenic presence. It is treated by medical practitioners with a variety of traditional antibiotics, either alone or in combination, and often for much longer courses than otherwise normally administered. One commonly used drug is PICC-administered intravenous Ceftriaxone - a cell wall inhibitor with a long half-life- which attempts to maintain MIC both temporally and compartmentally via administration directly to the heart. Accurate diagnoses of Chronic infection are complicated by the low bacterial load required for significant illness, and difficulties in isolating extant spirochetes from these patients.
Survival morphologies

Though decorin binding, and tissue sequestration allow the bacterium to establish persistent infection even in the presence of a strong immune response (Sellati et al., 1996) the spirochete seeks to disseminate beyond the initial infection site quickly to colonize secondary immune-secluded sites such as joints and central nervous system. The bacterium can hide in tissues less available to humoral immune response such as the central nervous system and cartilaginous sites, limiting drug and immune access in low circulation areas, and sometimes inducing auto-immune attack within the host. In addition to reducing antibiotic and immune access, this potentially provides an internal reservoir of infection (Groshong and Blevins, 2014; Hyde, 2017).

For instance, persistor cells are individual cells which appear identical to their population, but have one important difference: they reproduce on a different schedule. Since antibiotic compounds kill primarily during reproductive cycles, being off-cycle can have a protective advantage. It is important to note that these are individual cells, not full clonal lines, and that they seem genetically identical to their sister cells, meaning, these cells are not antibiotic-induced mutants, but a naturally-occurring portion of the population which confer resistance to pharmaceutical treatments through dormancy and regeneration. This delayed reproductive behavior has been documented in many bacteria, and seems to be related to ATP use (Sharma et al., 2015).

* Borrelia * also seems to persist in the face of high-stress environments such as antibiotic pressure and low nutrient availability is by encapsulation- having been documented to form round bodies, also called cell-wall deficient, or cystic forms. These cysts contain spirochetes within a glycolic boundary not susceptible to traditional antibacterial medications (figure 7). While thus invulnerable to antibiotics, the spirochetes multiply, representing a protected mini-reservoir of bacteria (Vancova et al, 2017). Cycling different

![Figure 7: Cyst formation](image)
antibiotics, or timed antibiotic hiatus are used by some physicians to address this survival technique, and some studies have shown success with anti-parasitic drugs used against round bodies.

The final documented survival morphology

*Borrelia* is known to enlist, is biofilm Sapi et al., 2012) (figure 8). This multi-layered microbial community contains many organisms and cofactors surrounded by a matrix of extracellular polymeric substances which inhibit access for outside attack by either host immune response or medication Biofilm communities represent a protective environment for bacterial propagation and a possible internal bacterial reservoir, impervious to many pharmaceutical treatments.

**Alternative hypothesis for post-treatment illness**

Though many believe persistent infection is the cause of continued illness after *Borrelia* exposure, others hypothesize changes made to the host system by the bacteria and/or the treatments are behind persistent symptoms. They call this continued illness Post treatment Lyme disease Syndrome, or PTLDS. There can be some overlap with Chronic Lyme disease theories, as PTLDS does not rule out the continued presence of *Borrelia* in some form, it just posits a systemic change instigated by the combination of *Borrelia* and/or its treatment. Suggested possible causes for this systemic change include epigenetic switching (turning on, or off specific genes linked to the symptoms), lasting damage due to the burrowing of *Borrelia* into body compartments such as the blood/brain barrier, gut endothelium, pericardium, or cartilage; and microbiome disruption from antibiotic killing of important commensal bacteria- resulting in dysbiosis and severe health imbalances.

**What is being done?**

Many effective pre-infection measures are being pursued to kill *Borrelia* before it reaches its accidental human hosts. Habitat treatments such as bait stations containing insecticidal food or bedding
materials for mice, or genetic alteration of mice to reduce their viability as reservoirs (Buc thal et al., 2019) are being actively pursued. Attempts to create an effective vaccine against Borrelia have so far fallen victim to its biochemical sleight of hand. Outer membrane proteins were early identified as good vaccine target prospects (Luft et al., 2002), and the original LymeRix vaccine was targeted specifically against OspA, a membrane protein upregulated in the arthropod host with the intent of teaching our immune system to destroy pre-dissemination spirochetes. Unfortunately, the speed of spirochete sequestration led to autoimmune attack on infected cartilage and heart tissue in some patients and the LymeRix vaccine was removed from the market (Nigrovic and Thompson, 2007).

Recent advances have allowed the construction of a novel chimeric protein composed of epitopes from the most common OspC variants in canines. Immune response to each of the epitopes in the constructed antigen seems to confer specific antibody production sufficient to counter initial infections (Schreterova et al., 2017). This new protein forms the basis of an effective canine vaccine which is being studied for adaptation both in human vaccines, and diagnostic immune assays.

However, even without new infections, there remains a significant reservoir of illness that must be addressed, so approaches able to treat existing infections are in high demand. A primary goal in this search is finding Borrelia-specific compounds that will kill or immobilize the spirochete without damaging the host microbiome. Possible methods include targeting the bacterium based on Borrelia-unique characteristics, or interfering with mobility or antigen switching to allow clearance by the host immune system.

Once the drug is delivered to the spirochete, however, it must still enter the cell. These ancient bacteria are evolved to exclude compounds dangerous to them, with an outer membrane effective at excluding hydrophilic compounds, and an inner membranes capable of excluding most hydrophobic compounds. Richter, et al, use broad-scale testing and characterization to find patterns in compounds successful against gram negative bacteria. They include: compound must be small, flat, inflexible, contain a primary amine with low steric hindrance, and have a high amphiphilic moment (Richter et al., 2017).
Drug development

During the 1950s, known pathogens were extensively tested against natural products, whose antimicrobial compounds are evolutionarily optimized for bacterial penetration and seemed a promising source of new antibiotic medications. But *Borrelia* was not isolated until 1981 (Steere et al., 1983) and therefore it was not tested as fully against naturally-occurring compounds as many earlier-discovered pathogens, leaving a target-rich environment.

Due to the difficulty in getting conclusive medical solutions to Lyme disease related illness, patients often seek treatment from less scientific sources. One common path is to use traditional medicine and herbal supplements. Many sufferers believe that these treatments are effective and report significant positive result during their use. In the context of previous natural antibiotic source, we chose to use anecdotal success of herbal treatments for Lyme disease as an initial selection filter to choose natural products we wish to screen for *Borrelia* inhibiting compounds.

Specific Aims:
In order to test my hypothesis, I propose the following

**Aim #1**
Identify effective approaches to test and detect efficacy of compounds against *Borrelia burgdorferi* in vitro.

**Aim #2**
Review potential natural producers of anti-*Borrelia* compounds from anecdotally effective Lyme disease treatments and extract natural product constituents based on their polarity.

**Aim #3**
Test extracts against *Borrelia*, and test effective anti-*Borrelia* compounds for specificity by screening against common gram positive and gram negative pathogens.
# Materials and Methods

**COMPOUND SOURCES:**

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<td>ABS</td>
<td>root &amp; leaves</td>
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<tr>
<td>Artemesia Botanicals, Salem</td>
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<tr>
<td>Elk Mountain Herbs</td>
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<td>StarWest Botanicals</td>
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<tr>
<td>Time Laboratories</td>
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**Table 1:** Plant sources for test compounds selected based on anecdotal success in treatment of Lyme Disease
Non-pharmaceutical plant products were chosen and tested for inhibition of GFP-tagged *Borrelia burgdorferi* based on their use and anecdotal success as Lyme disease treatments. For purposes of selection, internet sites and natural medicine protocols were used to identify herbal products suggested for Lyme disease treatment. Plant products identified as part of treatments with multiple anecdotal successes, which were found in more than one reference location, were chosen to test. This resulted in a list of 31 plant products.

Two plant-based products previously identified in scientific literature as *Borrelia* inhibiting (stevia and monolaurin) (Goc et al., 2017) (Theophilus et al., 2015) and one animal-based product (bee venom) (Socarras et al., 2017) were chosen as internal positive controls. Two plant products (dandelion and nettle) were included for investigation due to their status as preferential fodder for *Borrelia*-resistant deer. All test compounds were acquired according to Table 1.

**Extract preparation**

Extractions were performed on whole plant products, left un-sterilized to allow for plant-native bacterial populations which might be responsible for their anecdotal effectiveness, and conducted in a fume hood, at room temperature, using each of three solvents. Non-sequential extractions were performed on each vegetative product, with water, methanol, and hexanes to maximize compound extraction. 20mL of sterile deionized water, 0.2 micron filtered HPLC grade methanol (Fisher Chemical A452-4), or Fisher Chemical hexanes, methylpentanes 4.2%, (Fischer Chemical H292-4) were used as solvents to access polar, semi polar, and non-polar compounds. In glass tubes, 1 fresh gram of each plant material was soaked under agitation on a VWR standard analog shaker, shake level 4. After 24 hours, free solvent was removed, reserved, and 20mL fresh solvent was added to the same plant materials for 36 hours.

The two extractions were combined for each sample, and reduced to complete extract. Water supernatants were frozen on an angle to facilitate sublimation, and lyophilized to complete extract using a Freezmobile 25 EL lyophilizer. Methanol and hexanes solutions were evaporated to complete extract, at room temperature in fume hood. For testing, each sample was re-suspended with 4mL of its original solvent (10x concentration) and sonication was used to aid dissolution where necessary.
Bee Venom Collection:

Bees were donated by Noah Wilson-Rich of Boston Best Bees, Boston, MA and cooled to 4°C to produce stupor. It was necessary to remove venom sacs whole to eliminate contamination by body compounds known to be bacteriostatic. While cooled, bees were placed on an ice-filled platform of a stereoscope and decapitated before removing the venom sac with tweezers. Intact venom sacs were frozen at -80°C for storage. Photos were taken on Nikon smz800, level 3, 30x magnification (figure 9).

![Figure 9: venom sac collection from common bee (Apis apis). a. Bees were cooled and handled on an ice platform to maintain stupor. b. venom sacs were removed whole to avoid contamination.](image)

Preparation of extracts for liquid and semi-solid testing of *Borrelia* growth inhibition

For solid plating, methanol and hexane extracts only were used. Whatman grade AA 6mm paper inhibition discs were sterilized via UV exposure. 20µL of each resolubilized extract, or control solvent, was applied to individual discs and allowed to evaporate for 1 hour in a biosafety hood. Discs were stored, sealed, at room temperature for 4 weeks before plating in novel lawn-alternative method.

For liquid plating, 3 µL of each 10x concentrated extract or control solvent were placed in individual wells of 96well plates and evaporated in a biosafety cabinet for 1 hour at room temperature.
**BORRELIA GROWTH**

<table>
<thead>
<tr>
<th>organism</th>
<th>strain</th>
<th>notes</th>
<th>media</th>
<th>amendments</th>
</tr>
</thead>
<tbody>
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<td><em>Borrelia burgdorferi</em></td>
<td>BgGFP</td>
<td>WT+ GFP, retained by Kanamycin and Gentamycin</td>
<td><em>Borrelia</em>-friendly BSKII-based liquid or solid</td>
<td>rabbit serum (all) Gentamycin &amp; agarose (solid) Kanamycin 100uL/mL Gentamycin 50uL/mL (BgGFP only)</td>
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<tr>
<td><em>Borrelia burgdorferi</em></td>
<td>BflaB</td>
<td>nonmotile mutant, retained by Kanamycin</td>
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<td></td>
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<td>MG1655</td>
<td>gram neg, model, wild type strain</td>
<td>MHBroth II (cultures)</td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>WO153</td>
<td>gram neg, model, high accumulation mutant</td>
<td>MHBroth II (solid)</td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>HG003</td>
<td>gram pos model, wild type strain</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2- strains and media employed (Samuels, 1995)

**Initial Borrelia semi-solid growth curve**

Low passage isolates of *B. burgdorferi* strain B31 with an added GFP and flaB non-motile mutant *B. burgdorferi* were cultured in 5mL Barbour-Stoner-Kelly II (BSK-II) media (Sigma, St Louis, MO) supplemented with 6% rabbit serum and kanamycin at 100uL/mL and gentamycin 50uL/mL in GFP strains to maintain the plasmid containing the GFP cassette. Cultures were maintained in sterile 15 ml tubes and incubated at 34 °C with 5% CO₂, 3%O₂, and 13% relative humidity (Coy hypoxic glove box) for 6 days.

Spirochetes were counted by eye using three 6µL samples of each starting culture, wet-mounted on glass slides with cover slips via black field microscopy, Three fields each for each sample were counted, and the field counts were averaged. The starting culture was diluted to 1.00X10³ spirochetes per mL with fresh BSK-II amended liquid media, and incubated in coy hypoxic glove box at 5%CO₂, 3%O₂, 34°C, and 13% relative humidity for 10 days.

24 hours after the second inoculation, and repeating every 24 hours for 10 days, 6µL diluted culture was placed between 2x 2mL layers of BSK-II amended media containing 7% gelatin and 1.7% Agarose (SeaKem) on 6 well cell culture plates. For each time point, undiluted initial culture as positive control, media-only negative control, and three culture dilutions in triplicate, were plated. Plates were sealed and incubated at 34 °C with 5% CO₂, 3%O₂, and 13% relative humidity (Coy hypoxic glove box). All plates were counted for CFU by naked eye 10 days post-plating and the triplicate daily counts were averaged.

**Testing growth effects of herbal extracts using novel lawn-alternative semi-solid plating Method**

2mL layer of *Borrelia*-amended BSK-II semi-solid media was poured into 6-well cell
culture plates, and visually divided in three areas (figure 10). A 6mm inhibition disc soaked with plant extractions of either methanol or hexanes, or a control, was allowed to evaporate solvent under a fume hood, then placed in a labelled plate area. Six microliters of $1.00 \times 10^6$ spirochetes per mL *Borrelia* inoculate were administered to the center of each plate. A covering layer of 2mL semi-solid BSK-II *Borrelia*-amended agar was then poured over disc and inoculate layer and plates were sealed with parafilm M and incubated at 34°C with 5% CO$_2$, 3%O$_2$, and 13% relative humidity (Coy hypoxic glove box) for 10 days. Plates were photographed on Bio Rad chemi-doc MP fluorescent imaging system at 532excitation/528emission with .05sec exposure time, false-color green images produced, and also at room conditions with a camera phone.

Inhibition was read by eye, as zones of inhibition.

**Testing growth effects of herbal extracts using liquid plating**

Low passage isolates of *B. burgdorferi* strain B31 with an added GFP, were cultured by inoculating 5mL Barbour-Stoner-Kelly II (BSK-II) media (Sigma, St Louis, MO) supplemented with 6% rabbit serum and kanamycin at 100uL/mL, and gentamycin 50uL/mL to retain the GFP plasmid. Cultures were maintained in sterile 15 ml tubes and incubated at 34 °C with 5% CO$_2$, 3%O$_2$, and 13% relative humidity (Coy hypoxic glove box) for 6 days.

Spirochetes were counted by eye using three 6µL samples of each starting culture, wet-mounted on glass slides with cover slips via black field microscopy. Three fields each for each sample were counted, and the field counts were averaged. The starting culture was diluted to $1.00 \times 10^3$ spirochetes per mL with fresh BSK-II *Borrelia*-amended liquid media.
200µL of this inoculate was added to each well of 96well plate containing solvent-evaporated test compounds, and to wells for positive growth control, and antibiotic kill control. Media without inoculate was added to one well as a media control, according to the following plate map:

Plating was performed in duplicate, using 1x96-well plate per solvent. Plates were sealed using parafilm M and incubated in microaerophilic chamber, for 10 days, then and read for fluorescence on an electronic plate reader, using GFP settings: 532 excitation/528 emission. Change in growth from GFP-tagged B.burgdorferi negative control was calculated via: \[ \text{extract well growth minus negative control} / \text{Negative control} \] This calculation yielded percent difference from normal growth, with valence.

Counter-testing pathogenic lawns:

In order to test the compounds for specificity against Borrelia, gram positive (S. aureus) and gram negative (E. coli) were cultured by inoculating individual 5mL culture tubes containing Mueller Hinton II broth (MHB-II) liquid media with frozen E.coli strains MG1655 and WO153 and S.aureus strain HG003 and incubated under agitation at 27°C for several hours to approximately 0.6 OD. OD was measured via spectrophotometer. Cultures were diluted with MHB-II to 0.3 OD, and two Large rectangular plates per pathogen, containing 40mL Mueller Hinton II Agar (MHAII) were flooded with 40mL of either E.coli MG1655, attenuated E.coli WO153, or S.aureus HG003. Excess liquid was removed, and plates allowed to air dry in sterile hood. Plates were spotted with 3µL portions of the thirteen most active water-based extracts (methanol and hexanes extracts were not available) and incubated, without agitation, at 27°C for 24 hours. Counter testing was performed 3 months after Borrelia tests. Extracts were stored, solubilized at 4°C.
Results:

Plant product separation:

In order to generate plant extracts for testing against *B. burgdorferi*, I combined dried extracts with water, methanol, or hexanes. Plant products were observed during extraction, in the following ways: for floating, which informed access to solvent and could affect the success of the extraction; for cloudiness, to indicate either particulates or possible microbial growth; for film or bubbles as evidence of microbial growth that could give information about specificity; and for color, to look for correlations between color-forming compounds and antibacterial activity. Observations are tabulated in figure 13c.

![Image of extracted plant products](image.png)

### Table 1: Characteristics of Natural Product Extractions

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<td>29. Berberis</td>
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*Figure 13: Characteristics of Natural Product Extractions. Using 1g of 29 different plant materials, extracted in each of water, methanol, and hexane. Observations of extractive solutions were collected such as a- floating or cloudiness, or b. color. Tabulated observations, c, has columns for Float notes unsubmerged plant material; cloudy records suspended material or detritus; film/bubbles indicates possible bacterial growth, and color indicates solvent appearance. Yellow cells indicate positive fields.
Extraction of bee venom

Due to previous findings of bee venom as an effective antimicrobial against Borrelia (Socarras, 2017), this compound was selected for addition as a positive control. However, quantity of venom needed to reach MIC was 200 µL per test and the quantity of venom in each venom sac was 5 ± L, or less, not all sacs could be removed intact, and it involved killing the animal. It was therefore decided to halt collection after 50 animals, as the ecological cost was prohibitive. Therefore, I did not test bee venom in the Borrelia growth inhibition assays as originally planned.

Growth Curve Results:

In order to better understand Borrelia growth patterns, I performed a 10-day growth curve using both GFP-tagged B. burgdorferi and flaB B. burgdorferi, a non-motile mutant, in triplicate. Data for flaB mutant was inconclusive due to early contamination, but the non-motile and no spread morphology were noted. The motile, GFP-labelled bacterium showed normal exponential and stationary phase transitions (Figure 14) and demonstrated migratory plate distribution rather than surface creep. Radial growth from the point of spotting, with regular areas of clonal clumping, rather than even, topical coverage was observed (not shown). Due to these growth characteristics, I designed a

![Figure 14 Growth curve for GFP-tagged B. burgdorferi shows normal growth rate and phase transitions. In-vitro growth curves of GFP-tagged B. burgdorferi in solid Borrelia-optimized colorimetric media incubated under microaerophilic conditions. Starting concentration of 1.00X10^3 Counts of colony forming units at 36, 60, 84, 108, 132, 156, and 180 hours were performed in triplicate and enumerated by eye after 10 days incubation and graphed on a logarithmic scale. Error bars indicate standard deviation.](image)
novel lawn-replacement plating method for compound testing as an alternative to traditional pathogenic lawn growth inhibition assays impossible with this organism.

Bacterial pathogens are commonly tested for antibiotic inhibition by creating agar-based solid media with an even, topical, bacterial growth called a lawn. The bacteria is then exposed to possible antibiotic substances via direct spotting of extract (figure 12), or using paper inhibition discs (figure 15a) soaked with the test compound. This is impossible with Borrelia which prefers to move through, rather than on, a semi-solid matrix, and whose colony-formation is non-continuous. evenly- creating discrete clonal colonies rather than continuous distribution.

Figure 15: Novel semisolid plating method for growth inhibition of GFP-tagged B. burgdorferi in. a. Representative extract-soaked diffusion disks before use. Disks were added to BSK-II media, GFP-tagged B. burgdorferi was inoculated, and a second layer of BSK-II media was added. Plates were incubated for 10 days before examination by eye and camera phone (a&d) and by fluorescent imaging on Bio rad chemidoc MP fluorescent imaging system at 5532nm excitation/528nm emission.05sec exposure time (b&c). b. Possible zone of inhibition in dotted white outline, c. Representative 6-well plating fluorescent chemi-doc image d. Representative white-light plate with fungal growth seen in 2/3/4 (black fungus) and 11/12/13 (white fungus) wells.
Given my observations from the growth curve, where I saw centrally inoculated GFP-tagged *B. burgdorferi* spread radially in a punctuated but relatively even distribution, forming regularly distributed clumped colonies to the edges of the plate, I Therefore, a modification of the classic disk diffusion assay containing discs layered between media and using the bacterium’s documented movement pattern to create a lawn-alternative was implemented. Disks containing extracts reconstituted at 10x concentration were plated between 2 layers of semi-sold BSK-II *Borrelia*-amended media, with a central inoculation of the pathogen, incubated for 10 days in sealed, microaerophilic conditions, and the plates photographed both in room light and via fluorescent Bio rad chemi-doc MP fluorescent imaging system at 532nm excitation/528nm emission with .05sec exposure time, producing false-color images.

Zones of avoidance, represented by interference with normal *Borrelia* migration and colony seeding were expected, and, as seen in figure 15 b, c, were observed. The novel semisolid plating was successful as a test-of-concept, however the results were confounded by contamination and limited availability of test compounds. The issue of contamination was consistent throughout the experiment due to the very rich media and long incubation periods. An example of this contamination can be seen by fungal growth in figure 15 d. An anti-fungal media recipe which does not inhibit *Borrelia* has been perfected, and will be used in future iterations of this plating method. The black fungus seen in disc 2,3,4 of figure 15 d, however, is an interesting result allowed by the untreated media. This black fungus was not observed during extraction, but was present in all test containing hexanes extractives of plant product 2-andrographis.
Liquid Culture Test results:

Figure 16: 96 well plates containing extracts show differential growth of GFP-tagged *B. burgdorferi* in a. Representative water extracts have different colors which are not read as fluorescence, b. 10x extracts made from 30 plant sources were added to in 96 well and solvent allowed to evaporate. 200μL GFP-tagged *B. burgdorferi* in stock 10⁶ was added to each well, sealed with parafilm M, and incubated at 34°C with 5% CO₂, 3%O₂, and 15% relative humidity for 12 days. Fluorescence readings at 532nm excitation/528nm emission with quantify fluorescently tagged GFP-tagged *B. burgdorferi* in present in the specimen.

In order to test extracts, I prepared growth inhibition assays with each compound using all three types of extracts with a GFP-tagged *Borrelia*. After incubation, fluorescence in each well was recorded. Raw fluorescence data was checked. Raw fluorescence data was processed, subtracting *Borrelia*-only control (full growth), from well growth and dividing by full growth to obtain percent difference from full growth and valence \(\frac{\text{extract well growth-negative control}}{\text{negative control}}\). Negative values indicate reduction in growth as compared to control, positive values indicate increase in growth as compared to control (figures 17, 18, 19).
Liquid culture, water extracts: Twenty-seven water extracts were successfully tested against *Borrelia* in liquid media, and 24 of them showed action against the bacteria in the form of reduced growth. 13 of these extracts had at least half of the inhibition level shown by known effective antibiotic (positive control) with dandelion, nettle, and barberry demonstrating antibiotic-similar levels of inhibition, as compared to the positive control (Figure 17). Two compounds: Cat’s Claw and Teasel 1 increased GFP-tagged *B. burgdorferi* growth by 20% and 80% respectively, as compared to negative control. Rosemary and Wormwood failure to re-solubilize in water, and turmeric’s auto-fluorescence, made its results inconclusive.

Figure 17: Many water extractions inhibit GFP-tagged *B. burgdorferi* growth in liquid media: Net change in fluorescence when water extraction products are added to GFP-tagged *B. burgdorferi* during growth, in comparison to GFP-tagged *B. burgdorferi* native growth on duplicate 96 well plates. [(extract well growth-negative control)/negative control] is used to calculate percentage. Ax_b is antibiotic inhibition level (red), error bars represent standard deviation. * plant sources with missing data. N=2.
**Liquid culture, methanol extracts:** 30 methanol extracts were successfully tested against GFP-tagged *B. burgdorferi* in liquid media, and 24 of them showed action against the bacteria in the form of reduced growth. Five of these extracts: rosemary, clove, nettle, neem and stevia had at least 50% of the inhibition of doxycycline, and there was one notable extract: monolauren which exceeded antibiotic efficiency at 10x concentration by 11%. (Figure 18). Four compounds: cinnamon, Cat’s Claw, cordyceps, and panax ginseng caused increased growth by up to 15%, as compared to control, and turmeric’s auto-fluorescence again caused inconclusive results.
**Liquid culture, Hexanes extracts:** Where water and Methanol extracts showed overall good inhibition of *Borrelia*, with just a few extracts acting as growth enhancers, hexane extracts were more dichotomous. There were equal numbers of growth inhibitors and enhancers among hexanes extracts (Figure 19). 30 hexanes extracts were successfully tested against *B. burgdorferi* in liquid media, and 11 of them showed action against the bacterium. The strongest *B. burgdorferi* inhibitor found in the study was in the andrographis extract: showing 90% less fluorescence than the negative control. Teasel, dandelion and nettle each had at least half the action of antibiotic against GFP-tagged *B. burgdorferi*. On the other end of the spectrum, wormwood, stevia and cordyceps were all hearty growth enhancers 36%, 33%, and 20% respectively, and several other compounds enhanced growth notably, but to a lesser extent.

![Figure 19: Hexanes extractions both inhibit and amplify GFP-tagged *B. burgdorferi* growth in liquid media: Net change in fluorescence when hexane extraction products are added to GFP-tagged *B. burgdorferi* during growth, in comparison to GFP-tagged *B. burgdorferi* native growth on duplicate 96 well plates. ([extract well growth-negative control]/negative control) is used to calculate percentage. Abx is antibiotic inhibition level (red). Error bars represent standard deviation. N=2.](image-url)
Comparing Action in different fractions: When the relative actions of each plant’s three extractions, ostensibly representing three different subsets of extracted compounds based on polarity, are examined together, several plants seem to have contradictory responses (figure 20). Fennel, knotweed, cordyceps, stevia, cinnamon, panax ginseng, thyme, oregano, sarsaparilla, Siberian ginseng, teasel 1, and wormwood each have solute-variable inhibition and acceleration compounds. While this may make them less useful whole-source supplements, it does not invalidate them as drug-development sources for pharmaceutical purposes.

Figure 20: Comparison of GFP-tagged *B. burgdorferi* inhibition by plant compounds shows conflicting effect on GFP-tagged *B. burgdorferi* growth, ranked by level of water extract fluorescence. Blue-water extracted, green-methanol extracted, orange-hexanes extracted. Net change in fluorescence when extraction products are added to GFP-tagged *B. burgdorferi* during growth, in comparison to negative control on 96 well plates using [(extract well growth-negative control)/ negative control]. Abx is antibiotic inhibition level (red). Error bars represent standard deviation. * plant sources with missing data. N=2.
Counter testing results:

I chose the 13 most effective water extracts and spotted onto plates containing *E. coli* MG1655, attenuated *E. coli* WO153, or *S. aureus* HG003. Zones of inhibition were read by eye, based on antibiotic control, using a semi-quantitative 0-3 rating with 3 being antibiotic zone, and zero being no apparent zone of inhibition, but only a single extract (nettle) showed inhibition in counter testing. Counter testing was inconclusive, showing little inhibition, even from known gram-negative inhibitors like dandelion (10) grown in lawn and spotted with 13 best water hits at 10x strength show little inhibition, even from known gram-negative inhibitors like dandelion (10).

Note several dots show microbial growth in the area of extract addition rather than inhibition. Because the extracts seemed to have lost action, I did not proceed to HPLC separation and testing.

**Discussion**

This study sought to use anecdotal treatment success as an initial filter to identify natural products to test against *B. burgdorferi* for specific inhibition. Three extracts of each identified plant material, made using water, methanol, and hexanes individually, were exposed to fluorescently tagged *B. burgdorferi* and allowed to grow under conditions optimized for the bacterium. Bacterial growth was measured via fluorescence (in liquid media) and by estimated zones of inhibition (in solid media), and compared to positive and negative
controls to quantify changes in growth. In order to establish specificity, extracts effective in inhibiting \( B. \) 
\( burgdorferi \) were also exposed to common pathogens \( S. \) \( aureus \) (gram positive), and \( E. \) \( coli \) (gram negative) 
in lawn presentation. During the study, several plant sources warranting further investigation were identified, 
and a new method of testing compounds against \( Borrelia \) strains was piloted.

**On anecdotal usage-**

Herbs were an early source of medicine for humans, and persist in their importance to the 
pharmaceutical field. Antibiotic properties, often present in secondary metabolites, are the result of 
evolutionarily successful biological warfare and have become of greater interest as we see microbial 
resistance to previously identified antibiotics increase. Slightly higher than 10% of known plants have been 
used medicinally (Cowan, 1999), so finding an effective filter for choosing what to test is essential. 
Anecdotal success against Lyme disease proved to be just such a filter, providing 80-97% investigable hits 
between inhibitory and growth factors: Twenty nine out of thirty plants demonstrated inhibition of \( B. \) 
\( burgdorferi \) in at least one solvent extract (Table 3).

<table>
<thead>
<tr>
<th></th>
<th>count</th>
</tr>
</thead>
<tbody>
<tr>
<td>total extracts</td>
<td>30</td>
</tr>
<tr>
<td>anti-( Borrelia ) action in at least 1 fraction</td>
<td>29</td>
</tr>
<tr>
<td>contain both anti and pro-( Borrelia ) fractions</td>
<td>15</td>
</tr>
<tr>
<td>anti-( Borrelia ) action against all fractions</td>
<td>12</td>
</tr>
<tr>
<td>( Borrelia ) growth against all fractions</td>
<td>1</td>
</tr>
<tr>
<td>incomplete results</td>
<td>3</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>extracts</th>
<th>water</th>
<th>methanol</th>
<th>hexanes</th>
</tr>
</thead>
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<tr>
<td>total</td>
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<td>30</td>
<td>30</td>
</tr>
<tr>
<td>successfully tested</td>
<td>27</td>
<td>29</td>
<td>30</td>
</tr>
<tr>
<td>any ( borrelial ) inhibition</td>
<td>24</td>
<td>24</td>
<td>11</td>
</tr>
<tr>
<td>20% or greater ( borrelial ) inhibition</td>
<td>13</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>( borrelial ) growth</td>
<td>2</td>
<td>4</td>
<td>13</td>
</tr>
</tbody>
</table>

| percent extracts with \( borrelial \) inhibition | 89\% | 83\% | 37\% |
| percent extracts with 20\% or greater \( borrelial \) inhibition | 48\% | 21\% | 7\% |
| percent extracts with \( borrelial \) growth       | 7\%  | 14\%    | 43\%   |

Table 3 Anecdotal success proved to be an effective filter for \( B.\)\( burgdorferi \) inhibiting compounds: tally of extracts by solvent.
The action against *B. burgdorferi* from these plants was not uniform across solvents, meaning that different solvent extracts from the same plant sometimes had contrasting effects on the bacterium. This was true of 15 plants, and while such contradictory effects do not interfere with their pharmaceutical usefulness, because they can be purified in a lab setting, they could impact whole-plant applications, commonly employed in sub-clinical settings. A brief evaluation of cumulative action across the whole plant (summing the action of the three tested solvents) (figure 23) however, demonstrates a clear, strong, pattern of *B. burgdorferi* inhibition. While it is important to understand that the physiological effects of these herbs as oral treatments may not be cumulative in this way for any number of reasons, including bioavailability, tissue

![Bar chart showing whole plant (3 fractions), net effect](image)

**Figure 23:** Anecdotal Lyme disease treatments reliably predict plant sources which inhibit GFP-tagged *B. burgdorferi* in-vitro. Summed liquid testing results across water, methanol, and hexanes separation of 27 herbal plant sources.
passage, or deactivation by gastric enzymes, it demonstrates the success of using anecdotal Lyme disease remedies as a selection filter for compounds with *Borrelia* inhibition in-vitro.

An impressive twelve out of 27 fully tested plant sources showed inhibition of *B. burgdorferi* growth in all three extracts, while one (*Uncaria tomentosa*, or cat’s claw) was consistently growth-inducing, and three plants had incomplete test results due to inability to resuspend extracts after drying (rosemary and wormwood) or to intrinsic fluorescence (turmeric) which interfered with data collection.

On further investigation of the literature *U. tomentosa*, shows great promise in chemotherapeutic (Almeida et al., 2017) brain-plaque applications (Snow et al., 2019)and (surprisingly) against alternate *Borreli*al morphologies (Weiss, 2018), but its growth acceleration of the motile spirochetal form may confound its use. More investigation is needed in this area to understand how the inhibition of morphological presentation in *Borrelia* is linked to the acceleration of another.

Scientifically, compounds which enhance *Borrelia* growth could provide useful information about the metabolism of the pathogen, and molecular access pathways that could be accessed or inverted to kill it. Knowledge of what accelerates growth may contribute to our understanding of inhibiting growth as well. A more proximate use of growth accelerators of *B. burgdorferi* is to aid in recovering difficult-to-find spirochetes from mammalian samples, with a goal of understanding whether PTLDS is a consequence of continued spirochetosis (D’Onofrio, A. personal communication). Much further down the drug-development pipeline, an isolated *Borrelia* growth-enhancing compound could be used in combination therapies. A compound which denied the bacterium the ability to go dormant or form persisters could be a powerful treatment tool when administered with a companion antibiotic that kills (as most antibiotics do) during the organism’s reproductive cycle.

From a biochemical perspective, *Borrelia* growth acceleration in some fractions will not preclude investigation of their inhibiting neighbors, as inhibitory compounds may be chemically identified and separated. In a sub-clinical treatment setting, however, findings of cat’s claw *U. tomentosa* and *Dipsacus fullonum* (commonly known as fullers Teasel) as growth enhancers are potentially very important. These two
herbs figure prominently in many of the popular anti-Lyme disease protocols and self-administering patients could be unwittingly promoting long-term infection or PTLDS by their use.

**Entrenchment** is a persistent concern with use of herbal remedies against Lyme disease. The list of tested anecdotal uses in traditional medicine were researched on several popular herbal databases (figure 24) and found to fall into three main categories: antimicrobial, anti-inflammatory, and blood sugar control. This is relevant because anecdotal treatment success is necessarily subjective—representing an alleviation of symptoms, but perhaps not clearing the infection and allowing the bacterium to become even more deeply established. For instance:

for example, relieving inflammation caused by immune reaction to *Borrelia* infection (Grab et al., 2005) could provide apparent relief while leaving the infection intact; or temporary reduction of *Borrelia* population via a strong, but short-lived, antibacterial compound can reduce bacterial load, and kill important commensal organisms, while leaving persisters to replenish the pathogenic population (Sharma et al., 2015); or the lowering of free blood glucose which downregulates *Borrelia* proliferation by signaling stress-induced protective morphologies (Vancova et al, 2017) could result in a temporary relief of symptoms while leaving the infection intact.

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Anti inflammatory</th>
<th>Bloodsugar control</th>
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</thead>
<tbody>
<tr>
<td>rosemary</td>
<td>birch bark</td>
<td>cinnamon</td>
</tr>
<tr>
<td>clove</td>
<td>peels</td>
<td>milk thistle</td>
</tr>
<tr>
<td>garlic</td>
<td>turmeric</td>
<td>barberry</td>
</tr>
<tr>
<td>cats claw</td>
<td>neem</td>
<td>(fennel)</td>
</tr>
<tr>
<td>Japanese knotweed</td>
<td>cleavers</td>
<td>ginseng</td>
</tr>
<tr>
<td>wormwood</td>
<td>cordyceps</td>
<td>stevia</td>
</tr>
<tr>
<td>oregano</td>
<td>fennel</td>
<td></td>
</tr>
<tr>
<td>andrographis</td>
<td>ginseng</td>
<td></td>
</tr>
<tr>
<td>marjoram</td>
<td>sweet wormwood</td>
<td></td>
</tr>
<tr>
<td>teasel</td>
<td>sarsparilla</td>
<td></td>
</tr>
<tr>
<td>thyme</td>
<td>nettle</td>
<td></td>
</tr>
</tbody>
</table>

**Table 4: traditional medicine uses of chosen anecdotal compounds**

**Chemical Considerations:**

The physical observations of plant products during extraction met with expected results in that water extractions were more likely to demonstrate evidence of bacterial growth (film or bubbles). Whether from the dried natural products or growth in non-sterile conditions, this makes sense due to the comparatively antibacterial action of methanol and hexanes solvents. Bacterial inhabitants of the dried plant products might
have been removed by UV sterilizing the materials before extraction, and that may indeed be done in future iterations of this research, however the presence of a powerful fungal killer in the andrographis gave the best antibacterial results of anything tested, proving an initial test useful.

Another extraction consideration in our study is repetition of compounds due to non-sequential extraction. Initial testing involved individual solvent extractions to maximize potential compounds, but future studies should perform serial extractions to help purify relevant extracts. Even discounting possible overlap of extraction between solvents (methanol could separate some of the same compounds extracted by water or hexanes), plants containing efficacy across multiple fractions- like dandelion and nettle, are most likely displaying multiple *Borrelia*-effective compounds, which is in-itself, an exciting finding.

Water extracts were more likely to have floating plant material in the form of whole plant (float) or particulate suspension (cloudy) (figure 13) which did not seem to be a product of trapped air as it was not seen in the less polar solvents. This is likely due to the higher density of water, and the hydrophobic effect on more polar plant oils. Methanol drew brighter, clearer colors from the plants, often associated with antioxidant compounds and hexanes extracts were usually pale.

Water extracts had the most *B. burgdorferi* inhibiting compounds, somewhat surprising due to the their high polarity, in light of the (Richter et al., 2017) study. Some of these are easy to corroborate in the current literature, for instance: Japanese knotweed has many bioactive compounds and antioxidants and is the primary source of resveratrol for commercial supplements (Lachowicz and Oszmianski, 2019), and dandelion, chosen for its role in the Borrelia-immune deer diet has had some interesting findings also: (Diaz et al., 2018; Kenny et al., 2015). Barberry has action against E.coli and yeast membrane, which makes it an interesting candidate for suppressing both the spirochete and round body morphological forms, (Malik et al., 2017; Zoric et al., 2017), and fennel (Nguyen et al., 2014) joins other oily plant products in being an expected inhibitor.

Methanol’s best acting extract was one of our known controls, Monolauren, which arrived in pellet form and is a byproduct of coconut oil, separated for commercial uses, was predictably insoluble in water, but surprisingly partially insoluble in hexanes. This fulfills the necessary characteristic of large amphiphilic
moment in (Richter et al., 2017) and might be expected to affect its ability to access gram negative-like double membranes to be increased. Indeed, I found in liquid testing results that methanol solutions of monolaurin were very effective inhibitors of *B. burgdorferi*, and this effect was similar to that observed by (Goc et al., 2017).

Stevia, another positive control, had mixed action in our study, and the paper which caused it to be chosen as a control, (Theophilus et al., 2015) used alcohol extracts of stevia, suspended in their solvent and added to a liquid media culture of GFP-tagged *B. burgdorferi*, while I applied solvent-suspended extracts to 96 well plates and allowed them to evaporate before application. In this context, the finding of a methanol extract of stevia as inhibitory is consistent with current literature.

Herbaceous oils generally considered to be anti-bacterial both anecdotally and in literature (Liu et al., 2017) (Bower et al., 2016; Nguyen et al., 2014) and thus the inhibition exhibited by rosemary and clove oils were expected results, while low action by oregano was unexpected, and garlic- high in sulfur and a documented broad spectrum antimicrobial (Van Loi et al., 2019) was notably missing.

Hexanes extracts were interesting in their equal distribution between excitatory and inhibitory action, I hypothesize that this would have to do with membrane access of nutrients, but it is an area worthy of further study. The stand-out result, which showed near 100% inhibition of *B. burgdorferi*, and over twice the inhibition of the antibiotic control, was the hexanes-hardy volunteer fungus from Andrographis. Recent work by (Roy et al., 2016) identifies a newly documented andrographis-related bacterium with strong antimicrobial action *Bacillus thuringiensis KL*. Compounds in the plant itself have also shown promise (Arifullah et al., 2013), but the actor in the dish seemed to be the fungal growth, so this is an area that needs further exploration.

* *Borrelia* is tough to grow*

Much work went into investigating plating and detection methods for *Borrelia*. Due to the high detection threshold ($10^6$) of the plate reader (BioTek Synergy H1); loss of fluorescent result resolution past log phase due to accumulated free GFP; lack of success with several cell-dyes and markers; and the tendency of flow cytometry to shred the spirochetes or clog through spirochetal clumping, alternate detection methods
were investigated. Colorimetric agents were added to *Borrelia*-optimized plating but lacked specificity or ability to quantify results.

Commonly, pathogenic bacteria are grown in lawn presentation and dotted with antimicrobial compounds, then incubated and checked for zones of inhibition. However, due to Borrelia’s inter-media growth preference, migratory rather than creeping growth morphology, and colony-clumping growth behavior, traditional lawn presentation and dotting is not successful. Observations of GFP-tagged *B. burgdorferi* growth pattern on solid media led to development of Borrelia lawn-alternative, or novel semi-solid plating. In growth curve plating, GFP-tagged *B. burgdorferi* placed in the center of a plate was observed to migrate to the outer edges leaving spotty, but relatively uniform coverage throughout the media. Capitalizing on its pattern of migration, the idea of embedding test extracts within the semi-solid media was implemented. In order to allow presentation of the extract independent of the antimicrobial solvents (methanol and hexanes), paper inhibition discs were used. (Water extracts were not tested in this way due to lack of access.)

The pilot of this plating system was a qualified success, demonstrating avoidance zones around several extracts, and further optimization is in order. Committee member Dr. Vanja Klepac-Ceraj suggested adding Borrelia to the top plating layer to demonstrate true inhibition. While the theory is sound, implementation may be difficult due to the agarose/serum additives to the semi-solid agar which must remain hot until pouring and cannot be re-heated to liquefy without denaturing the blood additive.

Another stumbling block was the richness of the media. *Borrelia*- optimized media and long cultivation times make opportunistic microbial growth a constant difficulty. Contamination issues plagued both the growth curve and the novel semi-solid plating method inspired by it causing loss of almost all FlaB non-motile mutant *B. burgdorferi* growth curve results, day 9&10 of GFP-tagged *B. burgdorferi* growth curve results, and many of the semi-lawn plating results impossible to read. An antifungal/antibacterial additive recipe that does not interfere with *Borrelia* growth would be a necessary additive to the lawn-alternative plating approach.
One of the primary goals of this study was compound specificity to *Borrelia*, and the counter screen directed at establishing specificity demonstrated very little action against common gram positive and gram negative pathogens. While this might have been interpreted as specificity, the tested compounds included several known to have action against *E. coli* and *S. aureus*, for instance 10 (Diaz et al., 2018; Kenny et al., 2015) dandelion, and these were among those which failed to show zones of inhibition. This finding cause the screen to be invalidated, and was interpreted as loss of action due to the multi-month gap between resuspension and counter testing. In future iterations, ideally, counter testing would be completed at the time of *Borrelia* testing.

To pursue the findings of this study, future sequential multi-solvent extractions and separation by characteristics such as polarity and size, using resins, liquid-liquid extraction, addition of proteinaseK, and other known methods of purification should be employed on the top hits: from water- both the strong inhibitory hits of dandelion, nettle, barberry, fennel, Japanese knotweed, and neem from water extraction, and the growth hits: fuller’s teasel and cat’s claw. The andrographis fungus should also be investigated.

The resulting purifications should be re-tested against the original pathogen-set to isolate active compounds and isolated using High Performance Liquid Chromatography (HPLC) followed by fractional bioassays. Liquid Chromatography Mass Spectrometry (LCMS) should be used to establish novel mass, and Nuclear Magnetic Resonance (NMR) to elucidate structure of the thus-identified active compounds, and the compounds should be tested for general toxicity, hemolytic effect, and evaluated for MIC before administering to mouse models for in-vivo efficacy.

Long-term studies could continue to characterize our discovered compounds against multiple *Borrelia* strains, looking for phylogenic and evolutionary clues to fighting the pathogen, and differential effects that might make as well.
References


Lachowicz, S., and Oszmianski, J. (2019). Profile of Bioactive Compounds in the Morphological Parts of Wild Fallopia japonica (Houtt) and Fallopia sachalinensis (F. Schmidt) and Their Antioxidative Activity. Molecules (Basel, Switzerland) 24.


Almost the first *Borrelia* I ever grew