

EFFECTIVENESS OF *STEVIA REBAUDIANA* WHOLE LEAF EXTRACT AGAINST THE VARIOUS MORPHOLOGICAL FORMS OF *BORRELIA BURGdorFERI* IN VITRO

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Lyme disease is a tick-borne multisystemic disease caused by *Borrelia burgdorferi*. Administering antibiotics is the primary treatment for this disease; however, relapse often occurs when antibiotic treatment is discontinued. The reason for relapse remains unknown, but recent studies suggested the possibilities of the presence of antibiotic resistant *Borrelia* persister cells and biofilms.

In this study, we evaluated the effectiveness of whole leaf Stevia extract against *B. burgdorferi* spirochetes, persisters, and biofilm forms *in vitro*. The susceptibility of the different forms was evaluated by various quantitative techniques in addition to different microscopy methods. The effectiveness of Stevia was compared to doxycycline, cefoperazone, daptomycin, and their combinations. Our results demonstrated that Stevia had significant effect in eliminating *B. burgdorferi* spirochetes and persisters. Subculture experiments with Stevia and antibiotics treated cells were established for 7 and 14 days yielding, no and 10% viable cells, respectively compared to the above-mentioned antibiotics and antibiotic combination. When Stevia and the three antibiotics were tested against attached biofilms, Stevia significantly reduced *B. burgdorferi* forms. Results from this study suggest that a natural product such as Stevia leaf extract could be considered as an effective agent against *B. burgdorferi*.

Keywords: *Borrelia burgdorferi*, biofilms, persister cells, *Stevia rebaudiana*, antibiotic resistance

Abbreviations: ATCC – American type culture collection; BSK-H – Barbour–Stoner–Kelly H; CefP – cefoperazone; DapM – daptomycin; DoxC – doxycycline; EPS – extracellular polymeric substances; Log phase – logarithmic phase; PBS – phosphate buffered saline; PI – propidium iodide; PTLDS – post-treatment Lyme disease syndrome

Introduction

Lyme disease is a leading tick-borne multisystemic disease caused by the spirochete *Borrelia burgdorferi*. The bacterium is transmitted by Ixodes ticks, which could feed on white-footed mice, rodents, deer, and birds [1, 2]. In the United States, there are approximately 300,000 people diagnosed with Lyme disease each year [3]. The frontline treatment for Lyme disease is antibiotics such as doxycycline for adults and amoxicillin for children [4–8]. These antibiotics are found effective in most cases of patients diagnosed with Lyme disease [5–8]. However, according to the Centers for Disease Control (CDC), approximately 10–20% of the Lyme disease patients treated with antibiotics for a recommended 2 to 4 weeks experienced symptoms of fatigue, pain or joint and muscle aches [9]. In some patients, the symptoms even lasted for more than 6 months [9]. This condition was termed as “post-treatment Lyme disease syndrome (PTLDS)” or “chronic Lyme disease” [9].

The mechanism associated with this condition in patients remains unclear. Though not proven, there are a couple of suggested explanations, such as the inability of the immune system to completely clear *B. burgdorferi* persisters [10], or due to the presence of antigenic debris, which might cause immunological responses [11]. Another possibility of *Borrelia* evading the host immune clearance after antibiotic treatment is not well understood [12, 13].

Previous *in vivo* studies on mice, dogs, and nonhuman primates have shown that *B. burgdorferi* could not be fully eliminated by various antibiotics such as doxycycline, ceftriaxone, and tigecycline. Also, a recent study had demonstrated the presence of *Borrelia* DNA in mice following 12 months of antibiotic treatment [14]. However, the culturing of viable organisms in *Borrelia* growth media could not be achieved in these studies [14–17]. A recent study reported the presence of *Borrelia* DNA from a patient with PTLDS after antibiotic treatment [18]. Prospective clinical studies demonstrated no significant effective antibiotic therapy and failed to show evidence of the continued presence of

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B. burgdorferi in patients with long-term symptoms [19, 20]. Other trials using prolonged intravenous ceftriaxone treatment only improved fatigue symptoms [21]. In summary, findings suggest that conventional treatments may not completely eliminate *Borrelia* persisters.

The life style of *B. burgdorferi* is complex as it exists in different morphological forms like the spirochetes, spheroplast (or L-form), round bodies, and biofilms [7, 13, 22–25]. Several studies show that *Borrelia* can change into round bodies when conditions become unfavorable such as changes in temperature, high or low pH, starvation, antibiotic exposure, and/or even an attack from the immune system [7, 23–25]. *Borrelia* in these defensive forms becomes dormant, immobile, and remains in this morphological state until it finds favorable conditions to return to its spirochete form [7, 22, 24, 26]. The most effective hiding place proposed for *B. burgdorferi* is the recently suggested biofilm form [24]. Bacterial biofilms are organized communities of cells enclosed in a self-produced hydrated polymeric matrix or extracellular polymeric substances (EPS), which is a complex mixture of polysaccharides, lipids proteins, nucleic acids, and other macromolecules [24, 27]. This unique matrix protects the underlying cells from antimicrobial agents [24, 27]. Elimination of pathogenic bacteria in their biofilm form is very challenging since these sessile bacterial cells can endure the host immune responses and are much less susceptible to antibiotics or any other biocides than their individual planktonic counterparts [24, 27]. Biofilm resistance is based upon multiple mechanisms, such as phenotypic changes of cells forming in the biofilm, the expression of efflux pumps, and the presence of persister cells, which resist killing when exposed to antimicrobial agents [28, 29].

Recently, we provided evidence that *B. burgdorferi* is capable of forming biofilms *in vitro* [24]. The aggregation of spirochete and round body forms with several different protective layers which makes up the biofilm and extracellular polymeric substances (EPS) is proposed to be a significant factor in antibiotic resistance [24]. It is also reported that the biofilms have higher population of the persister cells, which could lead to the antimicrobial resistance portrayed by these forms [24, 29].

Our previously published results on the *in vitro* effects of doxycycline on *B. burgdorferi* showed that different morphological forms have unique sensitivities to antimicrobial agents [7]. A recent study by J. Feng et al. showed that doxycycline was effective in reducing the spirochetes but not the persisters of *B. burgdorferi* [30, 31]. Studies have also showed that doxycycline and amoxicillin could eliminate the spirochetal form of *B. burgdorferi*, but the dormant persisters/biofilm-like aggregates/microcolonies were not susceptible to these antibiotics [7, 31]. Therefore, there is an urgent need to find effective agents, which can target/eliminate all the morphological forms of *Borrelia*.

Natural antimicrobial agents, which have been used for thousands of years, have been shown to be effective against various pathogens [32]. Many *in vitro* and clinical stud-

ies have demonstrated their effectiveness not only against *B. burgdorferi* but also against many other pathogens [33–38]. *Stevia rebaudiana* which belongs to the Asteraceae family is typically referred to as honey leaf or sweet leaf, and due to its natural sweetness, it is used as a natural substitute to synthetic sweetener [39–41]. The leaf extract of *Stevia* possesses many phytochemicals, which include austroinullin, β -carotene, dulcoside, nilacin, rebaudi oxides, riboflavin, steviol, stevioside, and tiamin with known antimicrobial properties against many pathogens [40, 42, 43]. The role of these compounds is mainly to protect the plant from microbial infection and adverse environmental conditions [38–43]. *Stevia* is also well known in traditional medicine for its use in treatment of many diseases like diabetes, high blood pressure, and weight loss [44, 45]. In a few clinical studies, it is reported that the phytochemical stevioside reduces blood pressure in patients experiencing mild hypertension and reduces blood glucose levels in type 2 diabetic patients [44, 45]. It was also demonstrated that the patients did not encounter any adverse effects from the use of stevioside [44, 45].

Considering the effectiveness of *Stevia* leaf extract in laboratory and clinical studies, we evaluated the antimicrobial potential of *Stevia* (whole leaf extracts) against the Lyme disease causing pathogen, *B. burgdorferi*, in a goal to eliminate all the different morphological forms *in vitro*. To effectively evaluate the whole *Stevia* leaf extract, we compared the antimicrobial effect of *Stevia* with antibiotics (doxycycline, cefoperazone, daptomycin) and their combination, which were recently found effective against *Borrelia* persisters.

Materials and methods

Bacterial culture conditions and media requirements

Low passage isolates (≤ 8) of *B. burgdorferi* strain B31 were obtained from the American Type Culture Collection (ATCC 35210) and were cultured in Barbour–Stoner–Kelly H (BSK-H) media (Sigma, St Louis, MO) supplemented with 6% rabbit serum (Pel-Freez[®], Rogers, AR). The antibiotic free cultures were maintained in sterile 15 ml glass tubes and incubated at 33 °C with 5% CO₂. For logarithmic (log) phase, 1×10^5 cells/ml were seeded in glass tubes and allowed to grow for 5 days. The effectiveness of the antimicrobial agents on the logarithmic phase was tested by inoculating 1×10^5 spirochetes/ml from the five-day grown culture (containing only spirochetes) in 90 μ l of BSK-H media in 96-well tissue culture plates (BD Falcon, Franklin Lakes, NJ), which were incubated for 5 days at 33 °C with 5% CO₂. The cells were treated with antimicrobial agents for 3 days after a two-day incubation period. Likewise, for the stationary phase, 1×10^5 cells/ml were seeded in glass tubes and allowed to grow for 7 days. The effectiveness of the antimicrobial agents was tested by inoculating 1×10^6 spirochetes/ml in 90 μ l of BSK-H media in a 96-well tissue culture plate,

which was incubated for 8 days at 33 °C with 5% CO₂. The persister cells in stationary phase were treated with antimicrobial agents for 3 days after 5 days of incubation. Biofilms were generated by inoculating 5 × 10⁶ cells/ml of *Borrelia* spirochetes in 1 ml of BSK-H media in four-well chamber slides (Thermo Scientific, Waltham, MA) or plastic/collagen-coated tissue culture 48-well plates (BD Falcon), which were incubated for 7 days at 33 °C with 5% CO₂. The treatment regime for the biofilms was the same as that for the stationary phase.

Compounds and antibiotic preparation

Different Stevia extracts manufactured by Nutramedix[®], Now[®], Sweet leaf[®], and Truvia[®] were purchased from health food stores in the USA and were labeled randomly as Stevia A, B, C, and D. The extracts A, B, and C were formulated by standard alcohol extraction method whereas extract D was purchased in a powder form dissolved in distilled water. Stevioside (Sigma) was prepared in 0.001% DMSO and further diluted in 1× phosphate buffered saline (PBS, 0.1 M, pH 7.4 from Sigma). The antibiotics doxycycline, cefoperazone, and daptomycin (Sigma) at a concentration of 10 µg/ml were prepared in PBS. All antimicrobial agents were sterilized using a 0.2-µm filter unit (EMD Millipore, Billerica, MA). The antibiotic solutions were aliquoted and stored at minus 20 °C.

Determining the protein concentrations of the antimicrobial agents

Since the concentration of the antimicrobial components in the Stevia extracts A, B, C, and D were unknown, the concentration units used initially involved the use of part of solute per 50 parts of solution (1:50 dilution). The protein content of antimicrobial agents was determined by Bradford assay (Sigma) using serial dilutions of 2 mg/ml of bovine serum albumin stock (Sigma) in PBS pH 7.4 as a standard. Bradford reagent was added to the antimicrobial agents according to the manufacturer's instructions and incubated for 10 min at room temperature with gentle shaking. The absorbance was measured at 595 nm using a BioTek spectrophotometer.

In vitro antimicrobial susceptibility testing of B. burgdorferi

The effective antibiotics and the antibiotic combination identified [30] were tested to compare and evaluate the effectiveness of Stevia on *B. burgdorferi*. As a positive control, doxycycline (10 µg/ml) was used. As a negative control, 1× PBS at pH 7.4 was used to dissolve antibiotics and 25% alcohol (Fisher Scientific, Pittsburg, PA) was used since all the Stevia extract contained the same alcohol diluent.

SYBR Green I/PI assay

To evaluate live and dead cells, standard SYBR Green I/propidium iodide assay (SYBR Green I/PI) was performed as previously described [30, 31, 46, 47]. To 1 ml of sterilized distilled water, 10 µl of SYBR Green I (10,000× stock, Invitrogen, Grand Island, NY) and 30 µl of propidium iodide (Thermo Scientific) were briefly mixed. The staining mixture (10 µl) was added to all the wells containing *B. burgdorferi* and was incubated in the dark for 15 min. The plates were measured using a fluorescent reader (BioTek FLx800) by setting the excitation wavelength at 485 nm and the absorbance wavelength at 535 nm (green emission) and 635 nm (red emission). The standard equation was determined from 1 × 10⁶ cells (logarithmic phase), 5 × 10⁶ cells (stationary phase), and 1 × 10⁷ cells (7 days and 14 days subculture). A live and dead population was prepared. For the dead cell population, the cells were killed by adding 300 µl of 70% isopropyl alcohol (Fisher Scientific). *B. burgdorferi* suspensions at different ratios of live/dead cells (0:10, 2:8, 5:5, 8:2, 10:0) were added to the wells of the 96-well plate accordingly. The staining mixture was added to each of the five samples, and the plate was read as above. Using least square fitting analysis, the regression equation was calculated between the percentage of live bacteria and green/red fluorescence ratios. The regression equation was used to calculate the percentage of live cells in each sample of the screening plate. Also, images of the treated sample were taken using fluorescent microscopy (Leica DM2500).

Total viable counts of B. burgdorferi

As a confirmation test, the SYBR Green/PI stained cultures were assessed for cell growth by directly counting live and dead bacteria using a bacterial counting chamber (Hausser Scientific, Horsham, PA) and fluorescent microscopy (Leica DM2500). As above, using least square fitting analysis, the regression equation was calculated between the percentage of live bacteria and green/red fluorescence ratios. The regression equation was used to calculate the percentage of live cells in each sample.

Long-term subculture experiments to assess viability of treated B. burgdorferi stationary phase culture

To assess if the treated stationary phase cultures can regrow in fresh media, 96-well plates were filled with 100 µl of fresh BSK-H media. From the treated stationary phase cultures, 1:75 dilution of treated stationary phase culture was added to a sterile 96-well plate (BD Falcon) containing 100 µl of fresh BSK-H media and incubated for 7 days and 14 days without antimicrobial treatment. Following incubation, the viability was assessed using the SYBR Green I/PI assay and direct counting method.

Crystal violet biofilm assay

The efficacy of the antimicrobial agents on biofilms was determined by quantifying the total biomass using crystal violet staining. Following incubation, the media was slowly discarded leaving behind the attached biofilms on the surface of the plate. The attached biofilms were collected by adding 500 μ l of PBS (0.1 M, pH 7.4) to the samples. All centrifugation steps were performed at $12,000 \times g$ at room temperature. Biofilms were pelleted for 5 min, and the supernatant was discarded. A volume of 50 μ l (0.01%) crystal violet (Sigma) was added to the pellet, and the mixture was incubated for 15 min at room temperature. The unbound stain was removed by pelleting the biofilms for 5 min and by discarding the supernatant. The pellet was washed with 200 μ l PBS and again pelleted for 5 min. The supernatant was discarded in addition to adding 200 μ l of 10% acetic acid (Sigma) to the pellet to release and dissolve the stain. The samples were then incubated at room temperature for 15 min. Biofilms were pelleted for 5 min prior to extracting the crystal violet stain from the biofilms, which were transferred to a 96-well plate, and absorbance was measured at 595 nm using a BioTek spectrophotometer.

Live/dead bacterial staining technique

To visualize the antimicrobial sensitivity of biofilms, the treated biofilms were stained using SYBR Green (Invitrogen) and propidium iodide (Thermo Scientific). The stains were prepared as per the SYBR Green I/PI assay protocol. To each well of the chamber slide, 5 μ l of the staining mixture was added and allowed to incubate in the dark for 15 min. The media was removed carefully not to disturb the attached biofilm. Slides were cover slipped, and images were taken using fluorescent microscopy (Leica DM2500).

Atomic force microscopy

To visualize the morphology of the antimicrobial treated biofilms grown on glass chamber slides (Thermo Scientific), the media was carefully removed without disturbing the biofilm. Contact mode AFM imaging in air was performed on a Nanosurf Easyscan 2 AFM (Nanosurf) using SHOCONG probes (APPNANO). Images were processed using Gwyddion software (Nečas and Klapetek).

Statistical analysis

Statistical analysis was performed using two-tailed Student's *t*-test (Microsoft Excel, Redmond, WA) and graphed using GraphPad Prism[®] 6.0 (La Jolla, CA). All experiments were performed a minimum of three separate times with at least three samples per experiment. Data was normalized to the control and presented as the mean \pm SD.

Results

In this study, we compared and evaluated the antimicrobial effect of *S. rebaudiana* whole leaf extract along with the antibiotics doxycycline, cefoperazone, and daptomycin on the different morphological forms (spirochetes, round bodies, and biofilms) of *B. burgdorferi*. We used a recently developed high throughput screening method [30, 31, 46] to assess the viability of the log phase and stationary phase *B. burgdorferi* after the different antimicrobial treatments (quantitatively and qualitatively) in conjunction with direct counting methods as described in our previous publication [7]. Similar to recent studies, we have evaluated both the log phase *B. burgdorferi* cultures which consist of individual spirochetes, the stationary phase cultures which consist of the persister cells, and the most resistant form of *B. burgdorferi*, the attached surface biofilms. Finally, atomic force microscopy was used in conjunction with the aforementioned methods, to visualize changes in the morphology in attached biofilms before and after antibiotic treatments.

Preliminary screening of different Stevia extracts

Here, we first evaluated four different commercially obtained Stevia extracts (3 alcohol extracts and one extract in powder form) and the purified Stevioside on *B. burgdorferi* to find the most effective agent for further studies using the SYBR Green/PI assay. The protein concentration of the four Stevia leaf extracts was determined by a standard Bradford assay and ranged from 1.2 μ g/ml to 1.9 μ g/ml. Stevioside was tested at concentration from 100 to 1000 μ g/ml, concentration as suggested from a previous study [42].

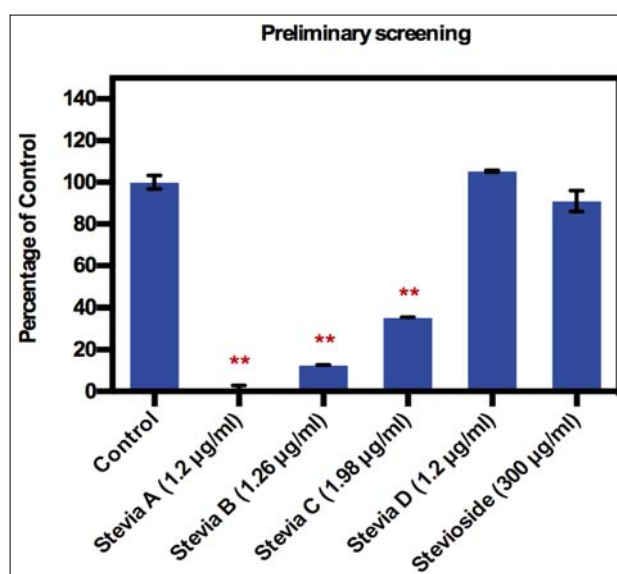


Fig. 1. Preliminary screening of four different extracts of Stevia and Stevioside on the stationary phase of *B. burgdorferi* after a three-day treatment using the SYBR Green/PI assay. $n = 3 \pm$ SD, * $p \leq 0.05$, ** $p \leq 0.01$ compared to the control

For the prescreening experiments, we have pretested all agents on the log and stationary phase *Borrelia* cultures at different concentrations using the SYBR Green/PI assay. Our results showed that Stevia A, Stevia B, and Stevia C alcohol extracted agents had significant effects on the viability of *Borrelia* cells, but Stevia D, powdered form, and Stevioside did not show any significant effect on both the log phase and the stationary phase cells. *Figure 1* shows a representative experiment demonstrating

that the alcohol extracted based Stevia agents (A, B, C) were the most effective against the *Borrelia* persisters, while the powder form of Stevia leaf extract (D) and Stevioside had no effect on those resistant cells. Furthermore, Stevia A showed the most promising effect in all experiments, and therefore, Stevia A was used in the subsequent experiments on the different morphological forms of *B. burgdorferi*.

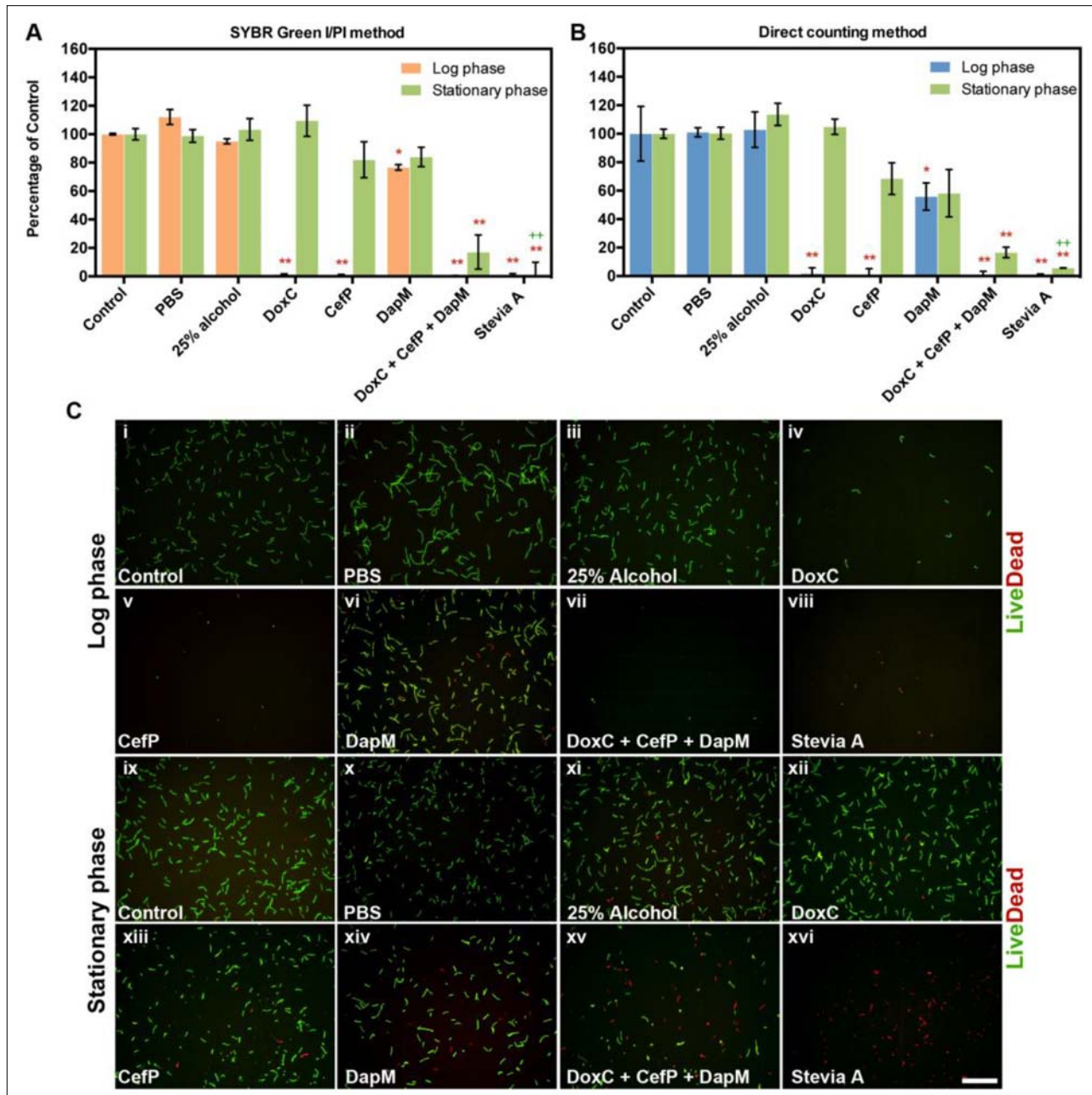


Fig. 2. Susceptibility of log phase (5 days) and stationary phase (8 days) *Borrelia burgdorferi* to antimicrobial agents after a three-day treatment determined by (A) SYBR Green I/PI assay, (B) direct counting of live and dead cells stained using a mixture of SYBR Green I and propidium iodide using fluorescent microscopy. (C) Representative live/dead images of log and stationary phase *B. burgdorferi* to antimicrobial agents taken at 200 \times magnification (Scale bar – 100 μ m). Doxycycline (DoxC) was used as a positive control. 1 \times PBS and 25% alcohol were used as negative controls, respectively. All antibiotics individually as well as in combination were used at a concentration of 10 μ g/ml. Stevia A was used at a concentration of 1.2 μ g/ml. $n = 3 \pm$ SD, * $p \leq 0.05$, ** $p \leq 0.01$ compared to the control. $n = 3 \pm$ SD, * $p \leq 0.05$, ** $p \leq 0.01$ (doxycycline compared to Stevia A). $n = 3 \pm$ SD, * $p \leq 0.05$, ** $p \leq 0.01$ (doxycycline compared to Stevia A). *Abbreviations:* doxycycline – DoxC, cefoperazone – CefP, daptomycin – DapM

Stevia as a potential agent in eliminating different forms of B. burgdorferi

To compare and evaluate the effectiveness of Stevia on *B. burgdorferi*, recently identified potential antibiotics and their combinations were tested along with Stevia A extract using the SYBR Green I/PI assay on both log and stationary phase cultures. Appropriate amounts of 1× PBS pH 7.4 and 25% alcohol were used as negative controls.

According to the SYBR Green I/PI assay and the live/dead images, treatment with 1× PBS or 25% alcohol did not significantly reduce the viability of the spirochete rich log phase culture and persisters rich stationary cultures compared to the control (Fig. 2A, and 2C: panels i–iii and ix–xi). Figure 2C: panels i–iii and ix–xi demonstrated that the control B31 culture, 1× sterile PBS, and 25% alcohol treated cultures comprised only live cells (green).

In the next set of experiments, we tested several previously reported effective antibiotics on *B. burgdorferi* such as doxycycline, cefoperazone, and daptomycin individually and in combinations [30, 31].

Doxycycline, as reported earlier [30, 31] was significantly able to reduce the viability of log phase *B. burgdorferi* by ~99% compared to the control (Fig. 2A, and 2C: panel iv). However, doxycycline treatment had no significant effect on the cells in the stationary phase cultures as observed by increasing proportion of viable cells after antibiotic exposure compared to the control (Fig. 2A, and 2C: panel xii).

Cefoperazone at 10 µg/ml, which was previously identified as one of the drugs with high activity against *Borrelia* persisters [30, 31], significantly reduced the log phase viability of *Borrelia* by ~99% (Fig. 2A, and 2C: panel v) but, in contrast, only reduced the stationary phase viability by ~18% compared to the control (Fig. 2A). A large population of live round body forms and a mixture of live and dead spirochetal cells were observed in stationary phase culture (Fig. 2C: panel xiii).

Daptomycin, one of the other drugs previously identified with potent activity against *Borrelia* persisters [30, 31], also significantly reduced the live spirochetal enriched log phase culture by ~23%, but it was less sensitive in reducing viable cells in the stationary phase (~16% reduction of live cells) compared to the control (Fig. 2A). Images demonstrated that cells treated with daptomycin, both log and stationary phase show more live cells than dead cells (Fig. 2C: panels vi and xiv) compared to control.

A recent study reported that the combination of doxycycline, cefoperazone, and daptomycin successfully eliminated both spirochetes and persisters [30]. Our data from this study also confirmed the effectiveness of these three-antibiotic combination on *B. burgdorferi* by significantly eliminating the live log phase spirochetes (100% effect) and also significantly reduced the viability of the stationary phase culture by ~86% (Fig. 2A, and 2C: panels vii and xv).

In these experiments, we also tested potential antimicrobial agent Stevia A as an individual agent and com-

pared its effectiveness to the individual antibiotics and the three-antibiotic combination. In Fig. 2A, and 2C: panels viii and xvi, Stevia A significantly eliminated the log phase spirochetes and the stationary phase persisters compared to the control (100% effect for both). The log phase and the stationary phase culture treated with Stevia A had only dead cells (Fig. 2C: panels viii and xvi).

In Fig. 2A, the effectiveness of doxycycline and the three-antibiotic combination was compared with Stevia A. A significant elimination (p value ≤ 0.01) in the persisters was found with Stevia A compared to doxycycline.

Direct counting confirms the effectiveness of Stevia A on the log phase spirochetes and stationary phase rich persisters

To further validate the effectiveness of the antimicrobial agents evaluated using the SYBR Green I/PI assay, direct counting was performed as described previously [7, 31].

According to the direct counting method, the negative controls, 1× sterile PBS and 25% alcohol, did not significantly reduce the number of viable cells in both the log phase and stationary phase compared to the control (Fig. 2B). While doxycycline significantly reduced the log phase viability of *Borrelia* by ~98%, it did not reduce the stationary phase viability compared to the control (Fig. 2B). Cefoperazone was also significantly able to reduce the viability of log phase *Borrelia* by ~99% (Fig. 2B), whereas cefoperazone in the stationary phase cultures was less effective (~32% reduction) in eliminating the persisters as observed by increasing proportion of remaining viable cells after antibiotic exposure compared to the control (Fig. 2B, and 2C: panel xiii). Daptomycin, significantly reduced the live spirochetal enriched log phase culture by ~44% and also significantly reduced the stationary phase by ~42% compared to the control (Fig. 2B). The three-antibiotic combination of doxycycline, cefoperazone, and daptomycin significantly eliminated the log phase culture and also the stationary phase culture by ~84% compared to the control (Fig. 2B). The potent antimicrobial agent, Stevia A, significantly eliminated the log phase spirochetes and significantly reduced the persisters by ~94% (Fig. 2B).

In Fig. 2B, the effectiveness of doxycycline and the three-antibiotic combinations was compared with Stevia A. A significant reduction (p value ≤ 0.01) in the persisters was found with Stevia A compared to doxycycline.

Effectiveness of Stevia A after a 7-day and 14-day subculture of antimicrobial treated B. burgdorferi

In the above-mentioned experiments, we have demonstrated the effectiveness of Stevia A on both spirochetes and the persisters of *Borrelia* and provided data that its significant effect is very comparable to the three-antibiotic combination reported previously [31]. To confirm the effectiveness of Stevia on the persisters, we performed a

7-day and 14-day subculture experiment in fresh BSK-H medium to observe if the persisters, if any, still left in the culture, could regrow in fresh medium assayed by the SYBR green I/PI and the direct counting methods.

As expected, the treatment with negative controls, 1× PBS and 25% alcohol, was able to successfully repopulate the media with predominating green live cells similar to the untreated B31 control after a 7- and 14-day subculture (Fig. 3A, and 3C: panels i–iii and ix–xi). The three-antibiotic combination and cefoperazone resulted in 5–7%

of viable cells after a 7-day subculture, but there was a significant 14% increase in the number of viable cells treated with cefoperazone and a 11% increase in viable cells treated with the three-antibiotic combination after a 14-day subculture (Fig. 3A, and 3C: panels v, vii, xiii, and xv). There was a significant 13% and 53% increase in viable cells from the sample treated with doxycycline after a 7-day subculture and a 14-day subculture, respectively (Fig. 3A, and 3C: panels iv and xii). The treatment with daptomycin, however, produced predominating live cells

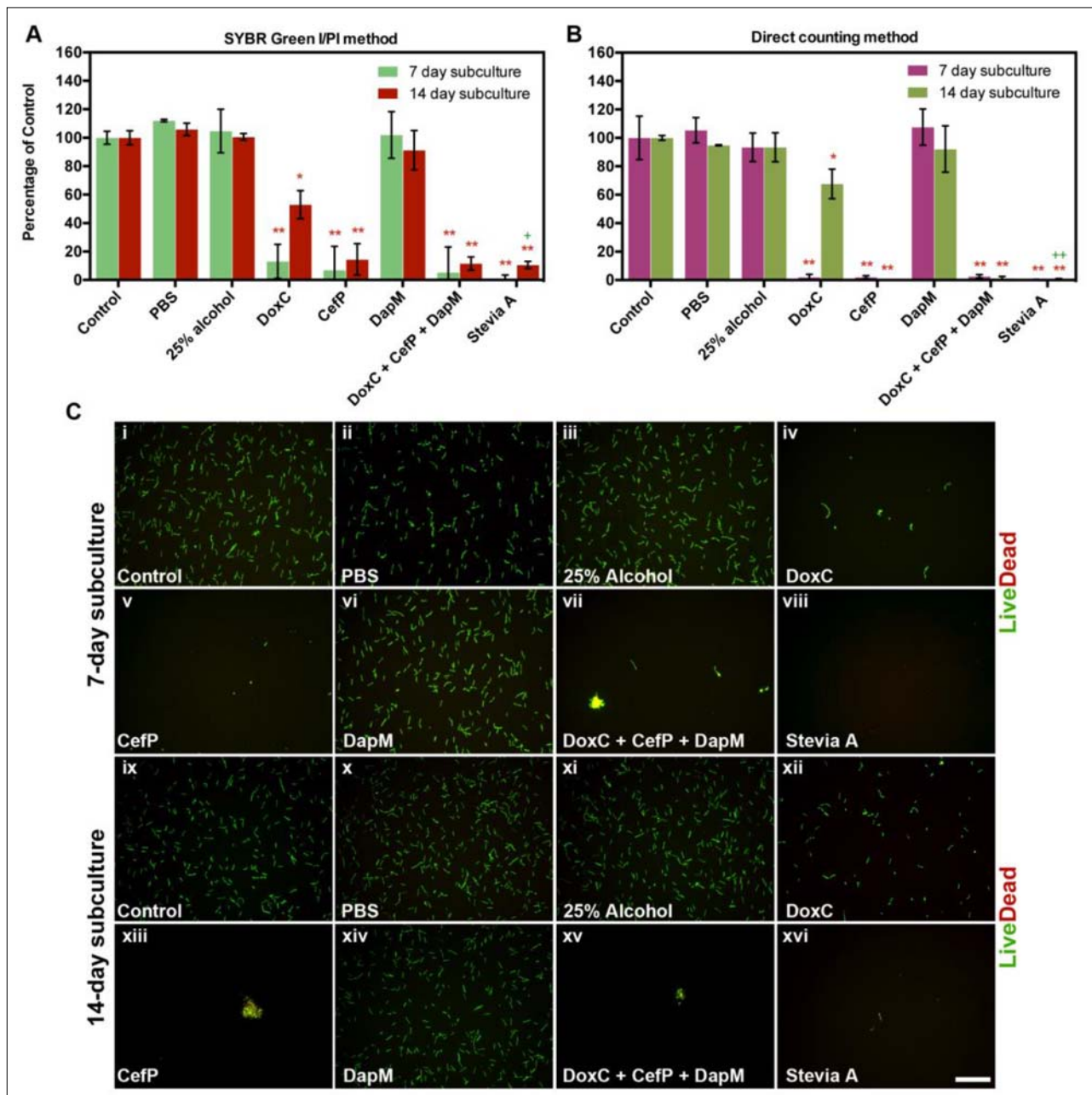


Fig. 3. A 7-day and 14-day subculture of 8-day-old *Borrelia burgdorferi* stationary phase culture treated with antimicrobial agents determined by (A) SYBR Green I/PI assay and direct counting of live and dead cells stained using a mixture of SYBR Green I and propidium iodide using fluorescent microscopy. (B) Representative live/dead images of a 7-day-old subculture on stationary phase *B. burgdorferi* treated with antimicrobial agents taken at 200× magnification (Scale bar = 100 μm). Doxycycline (DoxC) was used as a positive control. 1× PBS and 25% alcohol were used as negative controls respectively. $n = 3 \pm SD$, * $p \leq 0.05$, ** $p \leq 0.01$ compared to the control. $n = 3 \pm SD$, * $p \leq 0.05$, ** $p \leq 0.01$ (doxycycline compared to Stevia A). $n = 3 \pm SD$, * $p \leq 0.05$, ** $p \leq 0.01$ (doxycycline compared to Stevia A). **Abbreviations:** doxycycline – DoxC, cefoperazone – CefP, daptomycin – DapM

after a 7-day and 14-day subculture (Fig. 3A, and 3C: panels vi and xiv). Interestingly, there was no regrowth with the sample treated with Stevia A as there were only dead cells (100% elimination) after a 7-day subculture, and only a 10% increase in viable cells was observed after a subculture for 14 days (Fig. 3A, and 3C: panels viii and xvi).

In Fig. 3A, the effectiveness of doxycycline and the three-antibiotic combination after a 7-day and a 14-day subculture was compared with Stevia A. A significant reduction (p value ≤ 0.05) in the regrowth of *Borrelia* was found with Stevia A compared to doxycycline after a 14-day subculture.

Direct microscopic counting confirmed the SYBR Green I/PI assay and showed that drug-free controls and the negative controls (1× PBS and 25% alcohol) grew well in the 7-day and 14-day subcultures (Fig. 3B). Samples treated with doxycycline, cefoperazone, and the three-antibiotic combination produced ~2% viable cells after 7 days (Fig. 3B). Cefoperazone and the three-antibiotic combination produced ~1% viable cells after a 14-day subculture (Fig. 3B). However, there was an increase in viable cells by 68% treated with doxycycline after a 14-day subculture (Fig. 3B). Daptomycin treated cells were able to recover better than the other antibiotic counterparts with regrowth similar to the drug-free control in the 7- and 14-day subcultures (Fig. 3B). Samples treated with Stevia A after a 7-day subculture showed no signs of regrowth, and only 1% of viable cells were observed after a 14-day subculture (Fig. 3B).

In Fig. 3B, the effectiveness of doxycycline and the three-antibiotic combination in the direct counting method, after a 7-day and a 14-day subculture, was compared with Stevia A. Significant reduction (p value ≤ 0.01) in the regrowth of *Borrelia* was found with Stevia A compared to doxycycline after a 14-day subculture.

Stevia A significantly reduced Borrelia biofilms grown on plastic and collagen surfaces

Multiple morphological forms of *B. burgdorferi*, i.e. spirochetal form, round bodies, and biofilms have been observed to have different antimicrobial susceptibilities [7]. In our previous publication, we characterized the antibiotics sensitivity of the biofilm form of *B. burgdorferi* and found that it is the most resistant form [7]. In this study, to evaluate the effect of antimicrobial agents on *Borrelia* biofilms grown on plastic and collagen surfaces, we stained the antimicrobial treated biofilms with crystal violet to quantify the effectiveness of the different antimicrobial agents.

Biofilms grown on plastic surface and treated with negative controls, 1× PBS or 25% alcohol, did not show reduction in the biofilm masses compared to the untreated biofilm control (Fig. 4). Biofilms treated with doxycycline, cefoperazone, daptomycin, and the three-antibiotic combination showed significant increase in *Borrelia* biofilm mass compared to the drug-free control (Fig. 4). The

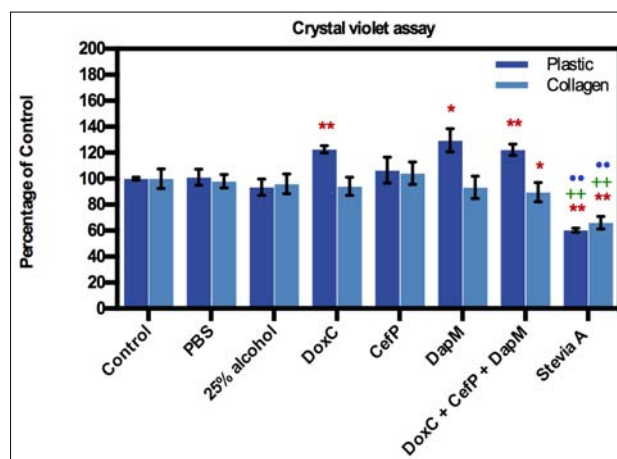


Fig. 4. Susceptibility of *Borrelia burgdorferi* biofilms grown on plastic and collagen-coated surface to antimicrobial agents after a three-day treatment determined by crystal violet assay. Doxycycline (DoxC) was used as a positive control. 1× PBS and 25% alcohol were used as negative controls, respectively. All antibiotics individually as well as in combination were used at a concentration of 10 µg/ml. Stevia A was used at a concentration of 1.2 µg/ml. $n = 3 \pm SD$, * $p \leq 0.05$, ** $p \leq 0.01$ compared to the control. $n = 3 \pm SD$, + $p \leq 0.05$, ++ $p \leq 0.01$ (doxycycline compared to Stevia A). $n = 3 \pm SD$, * $p \leq 0.05$, ** $p \leq 0.01$ (doxycycline compared to Stevia A). Abbreviations: doxycycline – DoxC, cefoperazone – CefP, daptomycin – DapM

treatment with Stevia A, however, significantly reduced *Borrelia* biofilms on plastic by ~40% compared to the control (Fig. 4).

Biofilms grown on collagen-coated surface show that the treatment with negative control, 1× PBS or 25% alcohol, did not show reduction in the biofilm masses compared to the control (Fig. 4). Treatments with doxycycline and daptomycin did not have any significant effect on *Borrelia* biofilm mass (Fig. 4). There was an increase in *Borrelia* biofilms grown on collagen when treated with cefoperazone, and in contrast, there was a 10% reduction observed in these biofilms when treated with the three-antibiotic combination compared to the control (Fig. 4). In addition, biofilms treated with Stevia A also showed significant reduction on collagen by ~34% compared to the control (Fig. 4). In order to assess the effectiveness of doxycycline and the three-antibiotic combination to Stevia A, the results in Fig. 4 showed that there was a significant reduction in the total *Borrelia* biomass grown on plastic and collagen surfaces by Stevia A compared to doxycycline and the three-antibiotic combination (p value ≤ 0.01).

Stevia A decreases viability in attached Borrelia biofilms

In order to directly observe the viability of attached biofilms after antimicrobial treatment, we stained the treated biofilms using a mixture of SYBR Green I and propidium iodide (live/dead stains). The *Borrelia* biofilms treated with 1× PBS and 25% alcohol were large and compact, mostly staining green, depicting that the biofilm was live

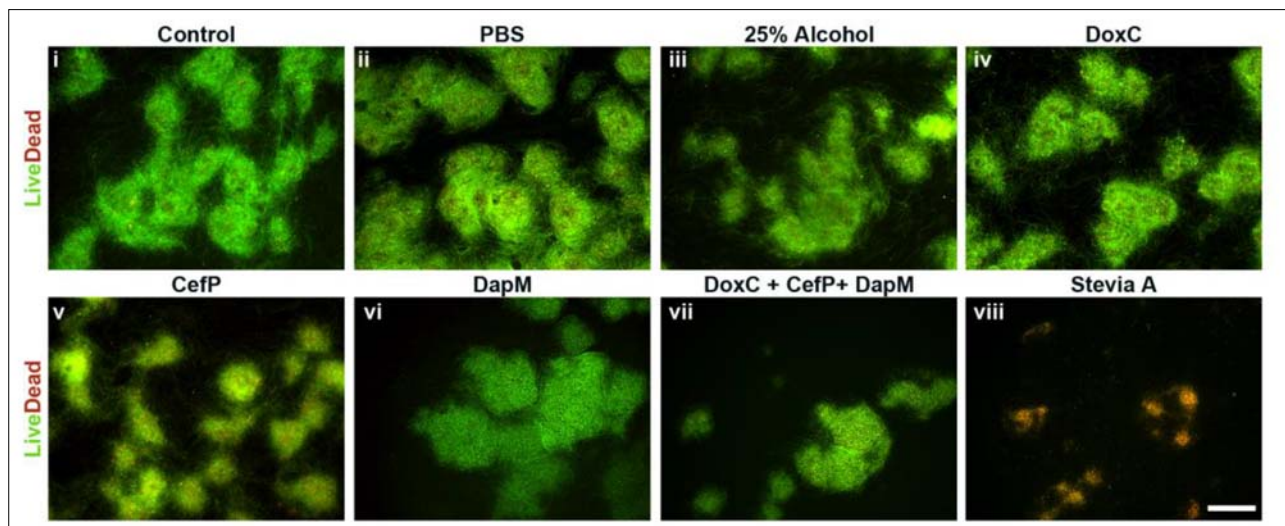


Fig. 5. Representative live/dead images of *Borrelia* biofilms treated with different antimicrobial agents followed by staining with SYBR Green I and PI dye mixture taken at 200 \times magnification. Doxycycline (DoxC) was used as a positive control. 1 \times sterile PBS and 25% alcohol were used as negative controls, respectively. All antibiotics individually as well as in combination were used at a concentration of 10 $\mu\text{g/ml}$. Stevia A was used at a concentration of 1.2 $\mu\text{g/ml}$. Scale bar –100 μm . *Abbreviations:* doxycycline – DoxC, cefoperazone – CefP, daptomycin – DapM

(Fig. 5: panels ii and iii). The biofilms treated with cefoperazone were significantly smaller compared to the biofilms treated with doxycycline, and both were green with small red spots indicating that they were alive (Fig. 5: panels iv and v). The biofilms treated with daptomycin were large and compact and stained mostly green with live cells

(Fig. 5: panel vi), whereas the biofilms treated with the antibiotic combination were made up with a mixture of live and dead cells with live cells predominating (Fig. 5: panel vii). *Borrelia* biofilm treatment with Stevia A stained predominantly red, depicting that the biofilm had mainly dead spirochetes and round bodies (Fig. 5: panel viii). The

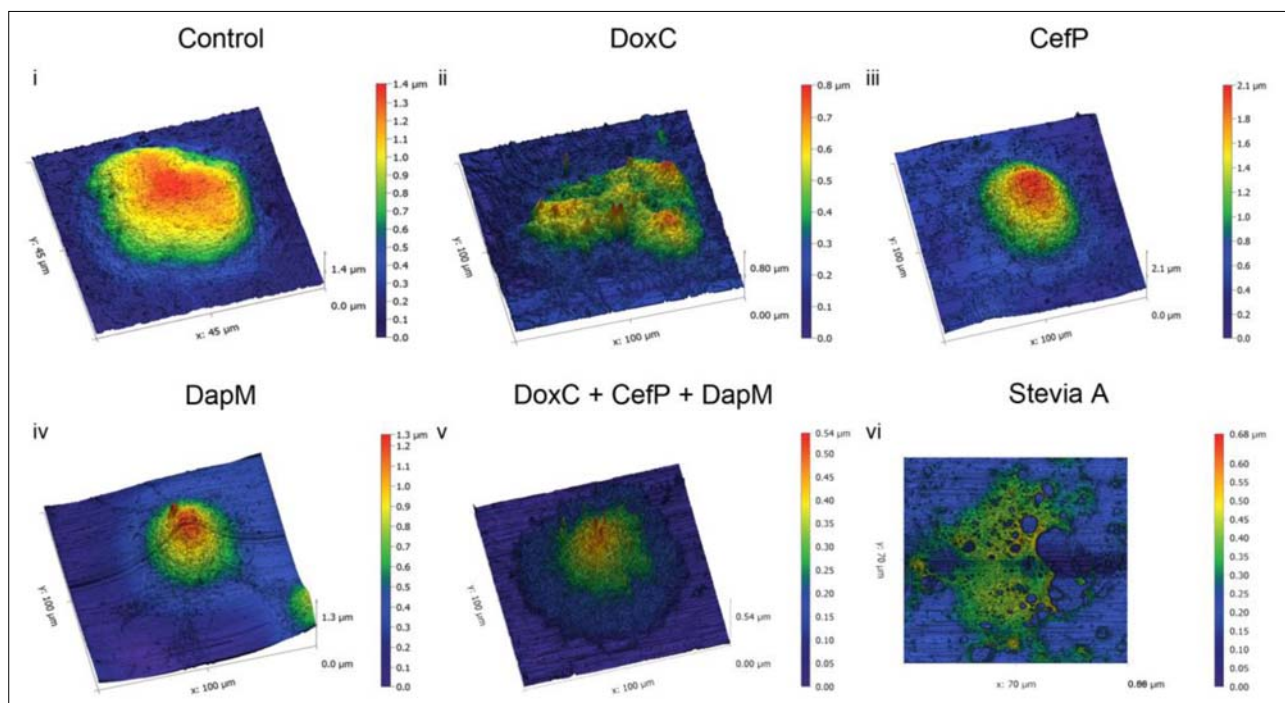


Fig. 6. Representative atomic force microscopy images showing the ultrastructural details of *Borrelia* biofilm before and after treatment with antimicrobial agents. The preparations of *B. burgdorferi* strain B31 biofilms on chamber slides are described in Materials and methods section. All biofilms were scanned at 0.4 Hz using contact mode and the individual Z ranges (height) are indicated next to each panel by means of a scale. The images were scanned using the Nanosurf Easyscan 2 software, and the images were processed using Gwyddion software. All antibiotics individually as well as in combination were used at a concentration of 10 $\mu\text{g/ml}$. Stevia A was used at a concentration of 1.2 $\mu\text{g/ml}$. *Abbreviations:* doxycycline – DoxC, cefoperazone – CefP, Daptomycin – DapM

morphology of the Stevia A treated biofilm was small and loosely packed compared to the biofilms treated with antibiotics (Fig. 5: panel viii).

AFM analysis shows loose morphology with biofilms treated with Stevia A

The atomic force microscopic images, which are 3D rendered and digitally colored for improved visualization, show the ultra structural features in the biofilms before and after treatment with antimicrobial agents. The red color shows the highest peak in the biofilms, which mainly indicates the potential presence of biofilm EPS matrix. The drug-free control had a very compact and rigid structure with notable EPS buildup (Fig. 6: panel i). The biofilms treated with doxycycline, cefoperazone, daptomycin, and the three-antibiotic combination were similar to the control having compact structure with more potential layers of EPS (Fig. 6: panels ii–v). Interestingly, Stevia treated biofilms have a very loose structure with the EPS layer almost not formed (Fig. 6: panel vi).

Discussion

In this study, we evaluated the antimicrobial potential of whole leaf Stevia extracts against the Lyme disease causing pathogen, *B. burgdorferi*. We compared the antimicrobial effect of Stevia with antibiotics (doxycycline, cefoperazone, daptomycin) and their combination, which were recently found effective against *Borrelia* persisters [31]. For this study, we have chosen to utilize a novel quantitative method developed by J. Feng et al. combined with our previously reported direct counting method [7, 30, 31, 46]. Findings from this study show that Stevia whole leaf extract, as an individual agent, was effective against all known morphological forms of *B. burgdorferi*.

In the preliminary screening experiment using different extracts of Stevia, we demonstrated a significant variation in the effectiveness of the alcohol extracts of Stevia (Stevia A, Stevia B, and Stevia C) on *Borrelia* persisters compared to the powder form of Stevia (Stevia D) and purified Stevioside which did not show any effect (Fig. 1). We also found that one of the Stevia compounds, Stevia A, significantly eliminated *Borrelia* persisters compared to other Stevia extracts (Fig. 1). This might be explained by the reported variations observed in the different phytochemical concentrations of different Stevia extracts, which resulted from the growing conditions and agricultural practices followed [47, 48]. Since Stevia A was effective in eliminating *Borrelia* persisters, Stevia A was chosen for further investigating the effectiveness on the different morphological forms of *B. burgdorferi*.

In the next set of experiments, we compared the effectiveness of Stevia A to the antibiotics and the three-antibiotic combinations found effective in recent reports

by J. Feng et al., [31] first to *Borrelia* spirochetes and then the persisters using the quantitative method developed by J. Feng et al. and E. Sapi et al. We observed that Stevia A, even at a lower concentration (1.2 µg/ml), could significantly eliminate the viability of early log phase and stationary cultures of *B. burgdorferi* using the SYBR Green I/PI method (Fig. 2A, and 2C: panels viii and xvi) which was also confirmed using the direct counting method (Fig. 2B, and 2C: panels viii and xvi). According to a report by J. Feng et al. (2015), daptomycin and cefoperazone, although effective against *Borrelia* persisters, could not completely eliminate the microcolony form of *B. burgdorferi* [30]. It was also observed from previous *in vivo* and *in vitro* studies that doxycycline was effective in eliminating the spirochetes but not the persisters of *Borrelia* [7, 16, 17, 30, 31]. However, J. Feng et al. also showed that the three-antibiotic combination (doxycycline, cefoperazone, and daptomycin) was very potent in eliminating the stationary phase rich persisters of *Borrelia* [30]. In this study, we provided evidence that Stevia A, as an individual agent, was capable of eliminating the spirochetes and the persisters of *Borrelia* similar to the reported three-drug combination treatment. Our data also showed that the antibiotics in combination on the persisters of *Borrelia* was indeed consistent with the previous study [30]; this result further confirms the effectiveness of Stevia A.

In our previous studies, we characterized the biofilm form of *B. burgdorferi* *in vitro*, and showed that it represents the most antibiotic resistant form [24]. It is also reported that biofilms attached to a surface are protected from antibiotics or other antimicrobial agents [28, 29]. In this study, we also evaluated the effect of all antimicrobial agents on attached *Borrelia* biofilms. Our results showed that Stevia A is very effective, in reducing attached *Borrelia* biofilm mass on both plastic and collagen coated surfaces by ~40% (Fig. 4) whereas the individual antibiotics actually induced the size of the biofilm mass (Fig. 4).

It was previously reported that certain antibiotics could indeed promote bacterial biofilm formation [49]. One potential explanation is that cells in biofilms are capable of protecting themselves from unfavorable antibiotic environment [24, 28] by developing several defensive mechanisms such as poor antibiotic penetration, phenotypic changes of cells forming in the biofilm, the expression of efflux pumps, and the presence of persister cells, which resist dying when exposed to antimicrobial agents [28, 29]. Persisters are a subpopulation of highly resistant cells, which are found among the normal cell population, and they are dormant and highly protected [28]. Studies show that small fraction of cells in biofilms were unaffected after prolonged antibiotic treatment [29, 53].

The obvious question on how Stevia could affect the highly resistant *Borrelia* biofilm warrants further investigation. In a study using a sugar alcohol, it was reported that xylitol acts as an antiplaque agent by disrupting the formation of biofilms in the oral cavity [54]. In another

study, they showed that xylitol affects the production of adhesive polysaccharides of *Streptococcus mutans* [55]. It was previously shown that sugars prime the uptake of antibiotics in *Staphylococcus aureus* and *Escherichia coli* [56]. Based on these previous findings, we hypothesize that Stevia could act as a sugar derivative, which might prime the uptake of the phytochemicals responsible for the antimicrobial effect and, thereby, disrupt the biofilm structure. In support to our hypothesis, we also showed that the ultrastructure of Stevia A-treated biofilm has very loose morphology with large shallow pits compared to the compact structure of biofilms treated with doxycycline, cefoperazone, daptomycin, and the antibiotic combination (Fig. 6: panels ii–vi).

To further confirm the effectiveness of Stevia, we performed long-term subculture experiments using the antimicrobial treated, stationary phase predominated with *Borrelia* persists by transferring a population of the treated cells into fresh culture medium to observe if viable cells can regrow after a 7-day and a 14-day period in the absence of antimicrobial agent. The natural antimicrobial agent (Stevia A) was shown to be effective with no regrowth of viable cells after a 7-day subculture and with only 10% increase in viable cells after a 14-day subculture. The effects of the three-antibiotic combination, when compared to Stevia A, regrew with 5% and 11% of viable cells as detected using the SYBR Green I/PI method and confirmed by fluorescent microscopy (Fig. 3A, and 3B: panels vii, viii, xv, and xvi). We also observed that *Borrelia* treated with doxycycline and cefoperazone, which did not show any significant effect against the persisters (Fig. 2A, 2B, and 2C: panels xii and xiii), recovering with only 13% and 6% viable cells after a 7-day subculture (Fig. 3A, and 3C: panels iv and v). One possible explanation for this phenomenon could be that antibiotic sensitivity might have been restored when bacteria are dispersed from a biofilm during favorable conditions [28].

Stevia leaf extract is a widely used sugar substitute [39–41, 57]; however, recent studies show that one of the major glycosides, stevioside, could have antimicrobial effect against *Bacillus cereus*, *Bacillus subtilis*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* [42]. These antimicrobial studies used a high concentration of purified stevioside, and in our study, we achieved similar antimicrobial effect against *Borrelia* by using a lower concentration of the whole leaf extract. Our data with purified Stevioside did not show any significant antimicrobial effect on *Borrelia* spirochetes and persisters compared to the whole leaf extracts of Stevia (Fig. 1). This finding suggests that other components within the Stevia whole leaf extract could have antimicrobial activity against *B. burgdorferi*, which are yet to be identified in future studies. In a good agreement with our findings, Stevia leaf extract has also demonstrated antimicrobial activity against pathogens such as *E. coli*, *S. aureus*, *Vibrio mimicus*, *Salmonella typhimurium*, *S. mutans*, *Bacillus subtilis*, *Shigella dysenteriae*, and *Vibrio cholera* [38–42]. The next question is whether Stevia could be safely used

as a therapeutic agent. Toxicological studies have shown that Stevia does not have mutagenic, teratogenic, or carcinogenic effects [57], and recent studies demonstrated its safety at high dietary intake levels [57–59]. In a study examining the mutagenicity of Stevioside and Steviol, it was noted that Stevioside at 10 mg/ml did not induce any mutation in *S. typhimurium* [60]. Apart from these studies, there are two important clinical studies based on the glycoproteins present in Stevia. In a randomized, double-blinded study on Chinese men and women experiencing mild hypertension, it was reported that the glycoprotein stevioside decreased the systolic and diastolic blood pressure and also improved quality of life without causing any adverse effects compared to the placebo [44]. In another study, the acute effects of stevioside in type 2 diabetic patients were analyzed [45]. Compared to the control group, stevioside reduces postprandial blood glucose levels in type 2 diabetic patients [45]. It was noted that both these studies used an encapsulated powdered form of stevioside and whole leaf extract that had been taken orally [44, 45]. It was also observed that one of the clinical studies used a whole leaf preparation, which contained 91% stevioside, 4% rebaudioside A, and 5% of other derivatives of stevioside [45]. The outcome from these clinical studies demonstrates that the patients did not encounter any adverse effects from the use of stevioside [44, 45]. Although the safeness of Stevia is widely studied, more *in vivo* studies are warranted before Stevia could be used as an antimicrobial agent for any infectious diseases.

Our future goal is to further investigate the individual components of whole leaf Stevia extract against *B. burgdorferi* and to identify the most effective component responsible for its significant antimicrobial effect. In conclusion, the overall antimicrobial effectiveness of Stevia A extract on the different morphological forms of *B. burgdorferi* was comparable to the combination of certain antibiotics. Although the results of this preliminary study cannot be extrapolated directly to clinical practice, further follow-up studies are necessary which can address the safeness of Stevia and to further identify the most effective component(s) against *Borrelia*.

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