Grapefruit Seed Extract is a Powerful *in vitro* Agent Against Motile and Cystic Forms of *Borrelia burgdorferi sensu lato*

Lyme borreliosis [1], caused by *Borrelia burgdorferi sensu lato*, may lead to long-term tissue infection, which may be difficult to cure. The outcome of Lyme borreliosis is highly dependent on the antibiotic treatment [2]. The observation of the ability of *B. burgdorferi sensu lato* to convert (and reconvert) to cystic forms [3–5] may explain why the infection sometimes is persistent and reactivating. Therefore, it might be important to eradicate all germative forms (not only the motile form) of the bacterium to obtain a proper treatment for Lyme borreliosis. Grapefruit-seed extract (GSE) contains bioactive flavenoids (e.g., hesperitin, resveratrol, and naringenin) and has been shown to possess anti-microbiological effect against bacteria and fungus [6, 7]. Many studies indicate that GSE is a substance whose therapeutic effect ranks equal to or better than other known anti-bacterial agents. Positive effects of GSE are decreased levels of TNF-α, Nuclear factor Kb, NO, protection of the gastrointestinal tract against mechanical stress, and has anti-allergic and other antioxidative properties [8, 9]. Naringenin, hesperidin and other citrus flavones have been found in plasma and tissue after ingestion [10]. *Lactobacillus* and *bifidobacteria* in the gut seems to be insignificantly affected by GSE [6], and no severe side effects have been observed. *B. burgdorferi sensu lato* has a gene for efflux mechanism which may be responsible for antibiotic resistance [11]. GSE is an efflux inhibitor, which can be used to enhance the activity of antibacterial agents [12]. For the reasons mentioned above it is reasonable to test the hypothesis that motile and cystic forms of *B. burgdorferi sensu lato* will be susceptible to GSE, and this is the aim of our study.

The bacterial strain used in our experiments was *B. afzelii ACA-1*. Production of mobile spirochetes and cystic forms was performed according to our previous procedure [13].

Grape fruit seed extract 33% (Citrosept; Cintamani Europe AS, 2071 Råholt, Norway) was diluted in distilled water, sterile filtered by a 0.2 µm filter, and diluted geometrically in 5 ml Nalgene tubes from 0.33%–0.00064% in 2 ml of diluted BSK-H medium (dilution 1:100 in distilled water). The control was diluted BSK-H. Two ml suspension of cystic forms at an age of 1 h was added to each of the tubes giving a final GSE concentration of 0.165%–0.00032%.

Susceptibility testing of mobile spirochetes to GSE was performed in a final dilution of GSE from 0.165% to 0.00032% in BSK-H medium. Forty microliter of 10^7/ml bacteria in logarithmic growth was added, making the final volume 4 ml in each tube. One control with only BSK-H was used. To examine if GSE could prevent the conversion of mobile spirochetes to cystic forms, testing was also performed in distilled water for 1 h at 34 °C. One control with only distilled water was used. Motile bacteria in distilled water and BSK-H medium were incubated aerobically.

The tubes with the mobile borrelia in BSK-H medium and the cysts in diluted BSK-H were examined by Dark Field Microscopy (DFM) (400×) after 1 h and 7 days to detect presence of eventual mobile spirochetes and intact cysts. Bacteria exposed to GSE in water were examined by DFM at 400× to examine the ratio of cyst/bacteria. Vital staining was performed on bacteria exposed to GSE for 1 week by mixing 10 µl of Live/dead *Bac*Light™ bacterial viability kit (Molecular Probes L-13152 Eugene, OR, USA) with 10 µl of the culture. This mixture was placed on a glass slide protected with a coverslip. The *Bac*Light-stained bacteria were examined by UV-microscopy (800×).

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Ø. Brorson
Dept. of Microbiology, Sentralsykehus i Vestfold HF, Tønsberg, Norway
Ø. Brorson
College University of Østfold, Fredrikstad, Norway
S.-H. Brorson
Institute of Pathology, Faculty Division Rikshospitalet, University of Oslo, Oslo, Norway
S.-H. Brorson *(corresponding author)*
The Pathology Clinic, Rikshospitalet-Radiumhospitalet Medical Centre, 0027 Oslo, Norway; Phone: (+47/23) 0714-94, Fax: -10, e-mail: s.h.brorson@labmed.uio.no

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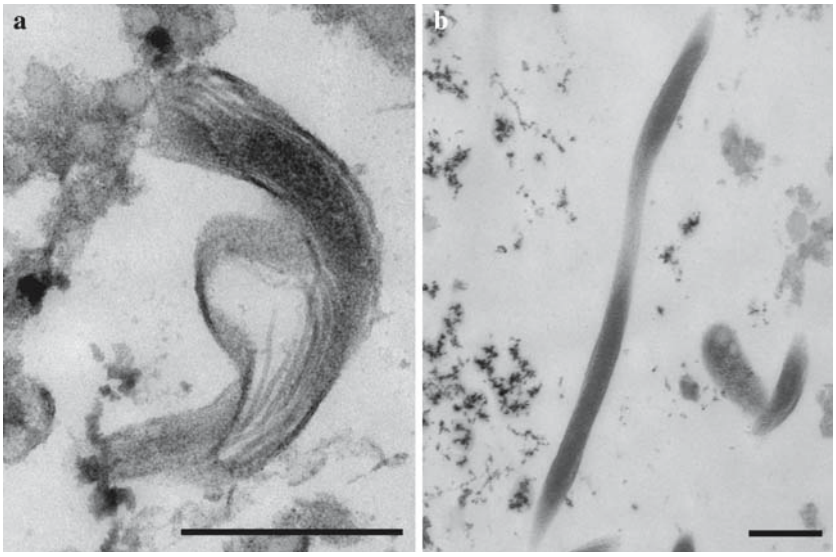

Figure 1. (a) Spirochetes incubated for 1 h at 34 °C with 0.165% GSE diluted in BSK-H medium. Only a very few pycnotic bacteria were present. Most bacteria were completely dissolved. (b) Spirochetes exposed to 0.041% GSE. The bacteria have a normal ultrastructure. TEM. Bar = 500 nm.

When the susceptibility testing for mobile spirochetes was performed in distilled water, the rate of conversion was strongly dependent on the GSE concentration. After incubation for 1 h at 34 °C the number of spirochetes converted to cysts ranged from none at GSE concentration of 0.165%–0.0052%, 10% at 0.0028%, 20% at 0.0013%, 95% at 0.00064%, and > 95% in the control when examined in DFM. By TEM, the dilution of 0.0013% showed a very few cysts; the dilution of 0.00064% showed many normal cysts but not as many as in the control.

Susceptibility testing of normal mobile borrelia exposed to GSE at 34 °C for 1 h revealed motile bacteria at concentrations ≤ 0.01%. After 5 weeks of incubation in fresh BSK-H medium, motile spirochetes were observed only at the dilutions ≤ 0.021%. By TEM some bacteria with normal appearance (compared to the control) were observed in the concentration of 0.041%, which is set to be the MBC (Figure 1). When the mobile spirochetes were exposed to GSE for 1 week at 34 °C in fresh BSK-H medium the estimated MBC was 0.0052% and MIC was ≤ 0.00032%. Four weeks of cultivation revealed 10^7 bacteria/ml in the 0.0026% dilution. However, BacLight™ showed green structures (green color indicates living organisms) only from the 0.0013% dilution. This corresponded well with results obtained by TEM.

Rupturing was observed by TEM and DFM for 100% of the 1 h old cysts which had been incubated in GSE from 0.165%–0.021%; for GSE-dilutions from 0.01%–0.00064% rupturing was observed for 90%–5%

The following cultures of spirochetes and GSE were examined by transmission electron microscopy (TEM) as earlier described [13]:

- motile spirochetes incubated for 1 week with GSE at a dilution of 0.0052%, 0.0026%, 0.0013% and a control without GSE in BSK-H medium,
- motile spirochetes incubated for 1 h with GSE at a dilution of 0.165%, 0.0825%, 0.0413%, 0.01% and a control without GSE in BSK-H medium,
- motile spirochetes incubated for 1 h with GSE at a dilution at 0.0413%, 0.0052%, 0.0013%, 0.00064% and a control without GSE in distilled water, and
- 1 h old cysts incubated for 1 h with GSE at a dilution of 0.021%, 0.01%, 0.0052%, 0.0013%, 0.00064% and a control without GSE.

GSE-exposed cultures were recultivated in BSK-H medium as earlier described [13] to confirm or invalidate the existence of viable bacteria. MBC of the mobile spirochetes was determined by recultivation of GSE-ex posed spirochetes, and the lowest GSE concentration where no growth occurred was set as the MBC value. The MIC value for mobile spirochetes was determined according to the lowest GSE concentration, which gave reduced multiplication when examined in DFM.

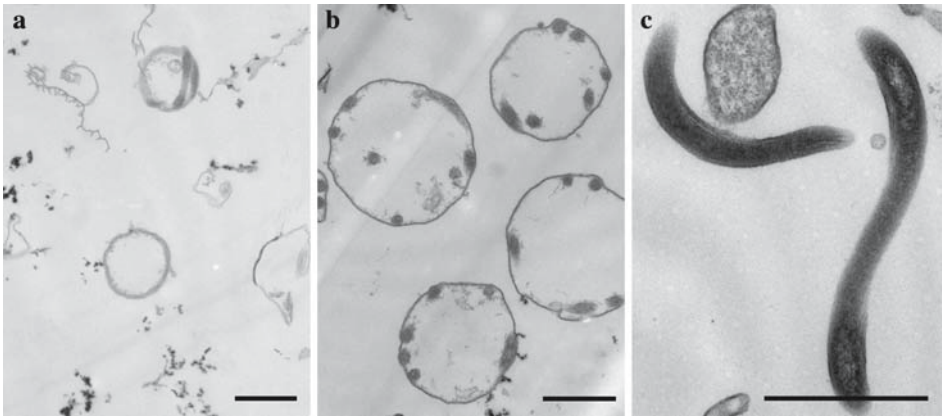

Figure 2. (a) One hour old cysts incubated for 1 h at 34 °C in BSK-H medium with 0.0013% GSE. A few normal and some dissolved cysts were present. (b) The same cysts as in A, but exposed to 0.00064% GSE. The number of normal cysts present was approximately the same as in the control. (c) The cysts in B were transferred into fresh BSK-H medium and incubated for 5 weeks in 34 °C. Many normal spirochetes were present. TEM. Bar = 1,000 nm.

(most rupturing for the less diluted GSE). For the negative control > 98% cysts occurred intact. When transferred to BSK-H medium, motile bacteria were observed after following incubation time: 14 days for the control and GSE dilution 0.00032%; 5 weeks for the 0.00064% dilution; no re-growth for higher concentrations (Figure 2). Therefore, the MBC was calculated to 0.0013%.

The highest GSE concentrations made the bacteria and cysts disappear completely, leaving only small uncharacteristic fragments; at lower GSE-levels the membranes showed herniation and disruption, and the contents had leaked out. The MBC was strongly dependent on the length of the incubation. GSE was very active even for very short incubation times, in agreement with previous results [7]. The MBC obtained by DFM for the motile bacteria agreed well with the TEM results. Presence of GSE reduced the conversion from spirochetes to cysts when the susceptibility testing was performed in distilled water. This study was performed *in vitro* and further studies are needed to demonstrate eventual effects *in vivo*. From our results it will be rational to test the hypothesis that a combination of GSE and antibiotics will be efficient in the treatment of resistant Lyme borreliosis.

Ø. Brorson, S.-H. Brorson

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