

1 Green tea and epigallocatechin-3-gallate are bactericidal against *Bacillus anthracis*

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12 Running Head: Green tea and EGCG kill *Bacillus anthracis*

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22 **ABSTRACT**

23 *Bacillus anthracis*, the etiological agent of anthrax, is listed as a Category A biothreat agent by
24 the United States Centers for Disease Control and Prevention. The virulence of the organism is
25 due to expression of two exotoxins and capsule, which interfere with host cellular signaling, alter
26 host water homeostasis, and inhibit phagocytosis of the pathogen, respectively. Concerns
27 regarding the past and possible future use of *B. anthracis* as a bioterrorism agent have resulted in
28 an impetus to develop more effective protective measures and therapeutics. In this study, green
29 tea was found to inhibit the *in vitro* growth of *B. anthracis*. Epigallocatechin-3-gallate (EGCG),
30 a compound found abundantly in green tea, was shown to be responsible for this activity, against
31 both the attenuated *B. anthracis* ANR and the virulent, encapsulated strain *B. anthracis* Ames
32 strain. This study highlights the antimicrobial activity of green tea and EGCG against anthrax
33 and suggests the need for further investigation of EGCG as a therapeutic candidate against *B.*
34 *anthracis*.

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43 INTRODUCTION

44 *Bacillus anthracis* is a Gram-positive pathogen that is the etiological agent of the disease
45 anthrax. The virulence of *B. anthracis* is attributed to three major factors: a γ -linked poly-D-
46 glutamic acid capsule and two binary toxins, Lethal Toxin (LT) and Edema Toxin (ET). The
47 capsule, encoded on plasmid pXO2, is believed to enhance virulence by its anti-phagocytic
48 properties (1). The plasmid pXO1 encodes the two A-B toxins; the toxins share a binding
49 partner, protective antigen, that pairs with either lethal factor to form LT or with edema factor to
50 form ET. LT cleaves mitogen-activated protein kinases and ET functions as an adenylate cyclase
51 to increase intracellular cAMP levels (2). These activities result in the dysfunction of key
52 signaling networks in myeloid cells leading to impairment of the host innate and adaptive
53 immune systems, and in late stages, damages to the cardiovascular system and liver (2). Human
54 infection occurs because of exposure to *B. anthracis* spores. Infection is acquired through
55 gastrointestinal, cutaneous or inhalational routes. Most natural infections occur through the
56 cutaneous route with minimal mortality, whereas inhalational anthrax is much more likely to
57 result in death (3). The prolonged environmental persistence of the spores combined with the
58 potential for their large scale dissemination, realized in the 2001 mailing of the Anthrax Letters,
59 has garnered the interest of the biomedical community and the public to improve current
60 prevention and therapeutic strategies against *B. anthracis* (3).

61 After water, tea is the most consumed beverage in the world. Although containing little
62 caloric value, teas have potent anti-mutagenic, anti-diabetic, anti-angiogenic, anti-inflammatory
63 activity (4, 5). Green and black teas are dried leaves of *Camellia sinensis*. After harvest, green tea
64 undergoes a heating process that inactivates the enzyme polyphenol oxidase. In contrast, black
65 tea is not heated, thus allowing the enzyme to function during tea processing. Thus, both green

66 and black teas contain similar amounts, but different compositions of simple polyphenols (4).
67 Whereas the polyphenol catechins predominate and represent 10 to 15% of the dry weight in
68 green tea, these simple polyphenols have been converted to their oxidized counterparts in black
69 tea (6). Importantly, of the four major catechins in green tea, EGCG predominates, representing
70 more than 50% of the total catechins (7).

71 Green tea has potent anti-microbial activity, which is attributed to EGCG because of its
72 relatively high abundance (8-10). EGCG demonstrates a broad range of anti-microbial activity,
73 with inhibitory effects on viral, bacterial and fungal pathogens (11). EGCG is especially
74 effective against Gram-positive bacteria (6, 12). The main mechanism of EGCG's anti-microbial
75 action has been postulated to be lipid bilayer damage (13). More recent evidence suggests that
76 EGCG also binds bacterial peptidoglycan resulting in compromise of the Gram-positive cell wall
77 (6). In addition, EGCG has been shown to reduce survival of *Listeria monocytogenes*,
78 *Mycobacterium tuberculosis* and *Legionella pneumophila* in macrophages by modulating cellular
79 functions (14-16). In the context of *B. anthracis*, EGCG inhibits LT function and protects
80 macrophages and rats from LT-induced death (17). However, to date, no data exist regarding the
81 effect of green tea or EGCG on the growth and viability of *B. anthracis*.

82 First, the effect of green tea was evaluated against an unencapsulated strain of *B.*
83 *anthracis* (ANR) under *in vitro* growth conditions. Next, we assessed the inhibitory activity of
84 EGCG against *B. anthracis* ANR in human blood. Last, the promising inhibitory and killing
85 activity against the unencapsulated ANR strain prompted us to test the activity of EGCG against
86 the virulent, encapsulated *B. anthracis* Ames strain.

87 MATERIALS & METHODS

88 **Bacterial strains and culture.** Vegetative unencapsulated *B. anthracis* ANR (pXO1+,
89 pXO2-) or encapsulated *B. anthracis* Ames (pXO1+, pXO2+) (U.S. Army Medical Research
90 Institute of Infectious Diseases) were grown overnight from spores in liquid cultures and used to
91 inoculate growth medium. All cultures were shaken at 37°C with 5% CO₂. All work with *B.*
92 *anthracis* Ames was performed under Biosafety Level 3 containment conditions.

93 **Growth medium preparation and blood collection.** The standard growth media used
94 were Luria Broth (LB) (Sigma, St. Louis, MO) or BBL Bovine Heart Infusion Broth (Becton
95 Dickinson, Sparks, MD) supplemented with 40% Fetal Bovine Serum (Hyclone, Logan, UT) and
96 0.8% NaHCO₃ (BHI/FBS). In some experiments, FBS was substituted with Bovine Serum
97 Albumin Fraction V (BSA) (final concentration 12 mg mL⁻¹) (Sigma, St. Louis, MO). Broth was
98 combined with tea, while maintaining a constant concentration of LB, BHI and/or FBS, and
99 inoculated 1:100 with an overnight culture of bacilli.

100 Whole blood samples were collected from healthy unvaccinated volunteers in vacutainers
101 containing sodium polyanetholesulfate (BD, Franklin Lakes, NJ). Research involving human
102 subjects was conducted in compliance with Department of Defense, Federal, and State statutes
103 and regulations relating to the protection of human subjects and adheres to the principles
104 identified in the Belmont Report (1979). All data and human subject research were gathered and
105 conducted under human use protocol # FY10-09. 5 mL of normal saline (0.9% NaCl) (control) or
106 saline containing EGCG (experimental) was added to 15 mL of whole blood for each condition.

107 **Tea and EGCG preparation.** Three tea bags (STASH © premium green or English
108 black breakfast tea, Portland, OR) were steeped in 120 mL boiling water for 3 min and sterilized
109 through a 0.2 µm filter. Epigallocatechin-3-gallate (EGCG) (Sigma, St. Louis, MO) was

110 solubilized in normal saline. Solutions of EGCG were prepared immediately prior to experiments
111 and sterilized through a 0.2 μm filter.

112 **Optical density (OD) measurements and Colony Forming Units (CFU) Counts.** OD
113 readings were taken at 600 nm using a Beckman DU530 Life Science UV/vis
114 Spectrophotometer. Cultures were serially diluted and plated either on Tryptic Soy or Sheep
115 Blood Agar Plates (Remel, Lenexa, KS) and incubated overnight at 37°C.

116 **Live/Dead Assay and confocal microscopy.** LIVE/DEAD *Baclight* Bacterial Viability
117 Kit (Life Technologies, Grand Island, NY) was used in accordance with the manufacturer's
118 protocol. Images were taken on the Zeiss 700 Laser Scanning Confocal Microscope System
119 using either a 40x/1.3 or a 100x/1.4 numerical aperture oil objectives lens with the pinhole set to
120 1 Airy unit. All images shown are maximum intensity projections and bars on images denote 20
121 μm in length.

122 **Statistical Analyses.** Microsoft Excel was used to analyze data and generate graphs.
123 Statistical significance was determined using an unpaired student's *t* - test at graphpad.com.
124 Error bars denote standard error of the mean (SEM).

125 RESULTS

126 ***Bacillus anthracis* ANR is killed in the presence of green tea.** In LB with 10% green
127 tea, *B. anthracis* ANR growth was significantly inhibited after 4 h, with a 2 log difference
128 between 10% green tea and the culture grown without tea (Fig. 1a). Black tea also inhibited *in*
129 *vitro* growth ($p = 0.08$) but to a lesser extent than green tea (Fig. 1a). Because green tea strongly
130 inhibited growth, the effects of time and increasing green tea concentration ranging from 10% to
131 50% were examined. Bacterial cultures subjected to green tea did not increase in turbidity,

132 whereas bacilli grown without tea did (Fig. 1b). Hence, green tea inhibits the growth of *B.*
133 *anthracis* ANR.

134 *B. anthracis* forms chains of bacilli (18). To assess if green tea resulted in killing of
135 individual bacillus within a chain, we performed a Live/Dead bacterial viability assay with
136 confocal microscopy imaging. Bacilli in chains were manually scored live or dead based on the
137 exclusion or inclusion of propidium iodide, respectively. Empty spaces within the chains, which
138 are cell wall remnants of dead bacilli, were also scored as killed. In the presence of green tea, a
139 decrease in the percentage of live bacilli was observed compared to the culture without tea (Fig.
140 1c & d). Interestingly, the effect of green tea on individual bacilli appeared to be random as live
141 and dead bacilli can both be seen on a single chain (Fig. 1d). Also, killing was concentration
142 dependent as increasing concentration of tea resulted in less live bacilli (Fig. 1c & d). Thus,
143 green tea inhibits the growth of *B. anthracis* ANR by a bactericidal mechanism.

144 **The presence of serum decreases the bactericidal activity of green tea.** We next
145 examined the activity of green tea in LB or BHI media that contained NaHCO₃ and FBS
146 (LB/FBS or BHI/FBS). Interestingly, we found a higher percentage of live bacilli were present in
147 LB/FBS + 50% tea versus LB + 50% tea without FBS (Fig. 2a). Similarly, a higher percentage of
148 live bacilli were scored in BHI/FBS with tea compared to BHI with tea (Fig. 2a). This suggests
149 that although green tea continues to inhibit bacilli growth in the presence of serum, FBS contains
150 compounds that interfere with green tea's bactericidal activity.

151 **Albumin is partially responsible for interfering with the bactericidal activity of**
152 **green tea.** Albumin is the most abundant protein found in serum and binds a variety of
153 endogenous and exogenous substances (19-21). To determine if albumin was responsible for

154 interfering with green tea's bactericidal effect, bovine serum albumin was substituted in place of
155 FBS (BHI/BSA). Compared to BHI alone, there were more live bacilli counted after growth in
156 BHI/BSA (Fig. 2b & c). However, there were less live bacilli in BHI/BSA than in BHI/FBS (Fig.
157 2b & c). Thus, BSA interferes with green tea's bactericidal effect. However, because BSA
158 cannot recapitulate the mitigating effects of FBS fully, it also suggests that there may be other
159 components in serum that can interfere with the anti-microbial activity of green tea. Another
160 possibility is that the addition of BSA or FBS provides *B. anthracis* with a richer medium for
161 growth thus increasing *B. anthracis* viability, even in the presence of green tea.

162 ***B. anthracis* ANR killing in BHI/FBS and blood by EGCG.** Because green tea's
163 antimicrobial activity is mainly attributed to the most abundant catechin EGCG, we next
164 explored the effect of EGCG against unencapsulated *B. anthracis* ANR. The average EGCG
165 concentration in brewed green tea is approximately 1-2 mM, but a range of 50µM to 4.5mM has
166 been reported (22-24). The maximum plasma concentration of EGCG that has been observed
167 following oral ingestion in humans is approximately 7 µM, but varies greatly (0.1 to 7 µM) and
168 is directly proportional to the amount of tea or extract ingested (25-27). Therefore, we tested the
169 effects of EGCG, ranging from 7 µM to 1.6 mM, on the growth of *B. anthracis* ANR in BHI
170 broth with and without FBS. Without FBS, there was significant inhibitory activity at
171 concentrations of 70 µM and greater (Fig 3a). Without FBS, 7 µM EGCG also inhibited growth,
172 albeit without statistical significance ($p = 0.09$) (Fig. 3a). In the presence of FBS, there was
173 significant inhibitory activity with 1.6 mM EGCG (Fig. 3b). 700 µM EGCG showed some
174 growth inhibition but this did not reach statistical significance ($p = 0.09$) (Fig. 3b). When
175 assessed by the Live/Dead assay, treatment with 1.6 mM EGCG showed significant killing of

176 bacilli at both 4 and 7 h in the absence of FBS. With FBS, the killing activity of EGCG was
177 mitigated at both time points (Fig. 3c).

178 EGCG reaches systemic circulation after green tea consumption (25). Therefore, the
179 effect of EGCG on *B. anthracis* ANR growth was also examined in human blood. In the
180 presence of 1.6 mM EGCG, a growth inhibition trend was observed in the blood at 6 and 24 h,
181 but this did not reach statistical significance (Fig. 3d). The difference in EGCG potency in media
182 with bovine (BHI/FBS) or human serum (blood) might be explained by the differences in serum
183 amounts or composition. Taken together, these data suggest that components in serum interfere
184 with the bactericidal activity of EGCG, but can be overcome with higher concentrations of
185 EGCG that saturate the inhibitory components in serum.

186 **Green tea and EGCG inhibit the growth of *B. anthracis* Ames.** The *B. anthracis*
187 capsule is a virulence factor that acts as a protective coat surrounding the bacilli preventing
188 phagocytosis and preventing molecules from reaching the cell wall of the bacilli (1). Thus, the
189 activity of EGCG against the encapsulated *B. anthracis* Ames was examined. First, the growth of
190 virulent bacilli was examined in BHI +/- FBS with 70 μ M or 1.6mM EGCG. Notably, even in
191 the presence of FBS, which was shown earlier to interfere with killing activity of green tea, 1.6
192 mM EGCG resulted in a three log decrease in CFU ml⁻¹ over the course of 6 h (Fig. 4a & b). For
193 cultures treated with 70 μ M EGCG, although no CFU count differences were seen, smaller
194 colonies resulted when grown compared to time-matched cultures that received no EGCG. (Fig.
195 4c). These data suggest that strain differences (ANR versus Ames) contribute to differential
196 susceptibilities to EGCG. EGCG was also evaluated against the growth of *B. anthracis* Ames in
197 human blood. At 24 h, there was a significant one log reduction in CFU ml⁻¹ in the presence of
198 EGCG compared to cultures without. As noted before, the lower potency of 1.6 mM EGCG in

199 blood (as compared to growth media) might be attributed to differences in the amount of
200 inhibitory components in human versus bovine sera.

201 **DISCUSSION**

202 Previous work has showed that black tea is bactericidal towards the unencapsulated,
203 avirulent Sterne strain of *B. anthracis* (28). Our work demonstrates the ability of green tea and its
204 constituent catechin EGCG to inhibit the growth of and kill the unencapsulated *B. anthracis*
205 ANR strain. Notably, our investigation also shows that green tea and EGCG exhibit bactericidal
206 activity against an encapsulated strain, *B. anthracis* Ames.

207 Mechanistic studies of EGCG's bactericidal activity against the Gram-positive
208 methicillin-resistant *Staphylococcus aureus* (MRSA) have revealed that EGCG binds to
209 peptidoglycan, resulting in impaired cell division and cell lysis (6). Thus, EGCG likely binds to
210 the cell wall of *B. anthracis* to cause lysis. Moreover, EGCG's activity against encapsulated *B.*
211 *anthracis* suggests that the capsule is permeable to EGCG or there may be areas devoid of
212 capsule, allowing the compound to reach the peptidoglycan beneath the capsule. Thus, in
213 addition to inhibiting the *B. anthracis* LT (17), EGCG is also bactericidal toward *B. anthracis*.

214 The highest concentration of EGCG measured in human plasma is 7 μM after ingestion
215 of a single 1600 mg dose. The large difference in amount of EGCG consumed and the
216 concentration found in systemic circulation is due to the breakdown of EGCG by intestinal
217 micro-organisms, poor absorption by the small intestines, and its active removal by efflux
218 transporters (29-31). Although we did not see effective killing at 7 μM , it may be possible to
219 achieve higher concentrations in blood if EGCG is administered intravenously. EGCG has been
220 shown to have minimal toxicity in animals and humans (32). Indeed, injection of EGCG to

221 achieve an estimated circulating dose of 100 μM in Fischer 344 rats allowed some to recover
222 from LT-induced toxicity. Moreover, rats and mice that were injected with EGCG alone did not
223 experience adverse reactions (17). Thus, intravenous administration of higher and repeated
224 dosing of EGCG should be evaluated for safety and efficacy in animal models of anthrax.

225 To increase the bioavailability of EGCG in the systemic circulation, alternative delivery
226 systems or chemical modification of EGCG should also be considered. A recent report has
227 shown that the nanoencapsulation of EGCG allows sustained release and site specific targeting
228 of the molecule (33). In addition, peracetylated EGCG has increased biological potency and
229 bioavailability, though once converted back to the parent compound, it undergoes the same
230 degradation and metabolism described previously (31). Alternatively, a different route of
231 administration that prevents EGCG from being rapidly metabolized or inactivated, such as a
232 nebulized form of EGCG, should be considered. Others have shown that the nebulized form of
233 tea catechins are safe and effective against methicillin resistant *Staphylococcus aureus* in the
234 respiratory tract of humans (34). Therefore, future studies should address alternative routes of
235 delivery for EGCG in order to avoid metabolic processes, chemical degradation and inactivation
236 by serum albumin and other blood constituents.

237 Further, it is important to explore the pharmacodynamic relationship of EGCG with
238 existing antibiotics. Importantly, synergy with another anti-microbial would reduce the required
239 amount of EGCG to achieve a bactericidal concentration *in vivo*. Zhao and colleagues have
240 shown synergy between EGCG and β -lactams (6). Although some strains of *B. anthracis* have
241 been reported as resistant, most strains are susceptible to β -lactams (35). Thus, EGCG might
242 synergize with β -lactams to enhance killing of *B. anthracis*.

243 Although EGCG has bactericidal activity against *Bacillus anthracis*, shown here in our
244 study, and against other pathogens (6, 36), it is important to note that other major catechins,
245 found in green tea, also have a similar function (36). Less abundant than EGCG, catechins such
246 as epigallocatechin and epicatechin gallate are also antibacterial agents. Moreover, the
247 bactericidal activity of green tea is likely the sum, if not from the synergistic combination, of the
248 individual catechins (36, 37). Thus, future work should explore the anti-microbial activity of
249 other green tea catechins individually and in combination with each other and EGCG. Finally, as
250 EGCG remains a promising drug candidate against *B. anthracis*, given its anti-bacterial and anti-
251 LT activity, and along with other pathogens, more efforts should be directed towards overcoming
252 some of EGCG's pharmacokinetic challenges.

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257 **DISCLAIMER**

258 The opinions, interpretations, conclusions, and recommendations are those of the authors
259 and are not necessarily endorsed by the U.S. Army. Research involving human subjects adhered
260 to the principles identified in the Belmont Report (1979) and, unless certified as exempt, was
261 conducted in accordance with an IRB-approved protocol and in compliance with Department of
262 Defense, Federal, and State statutes and regulations relating to the protection of human subjects.

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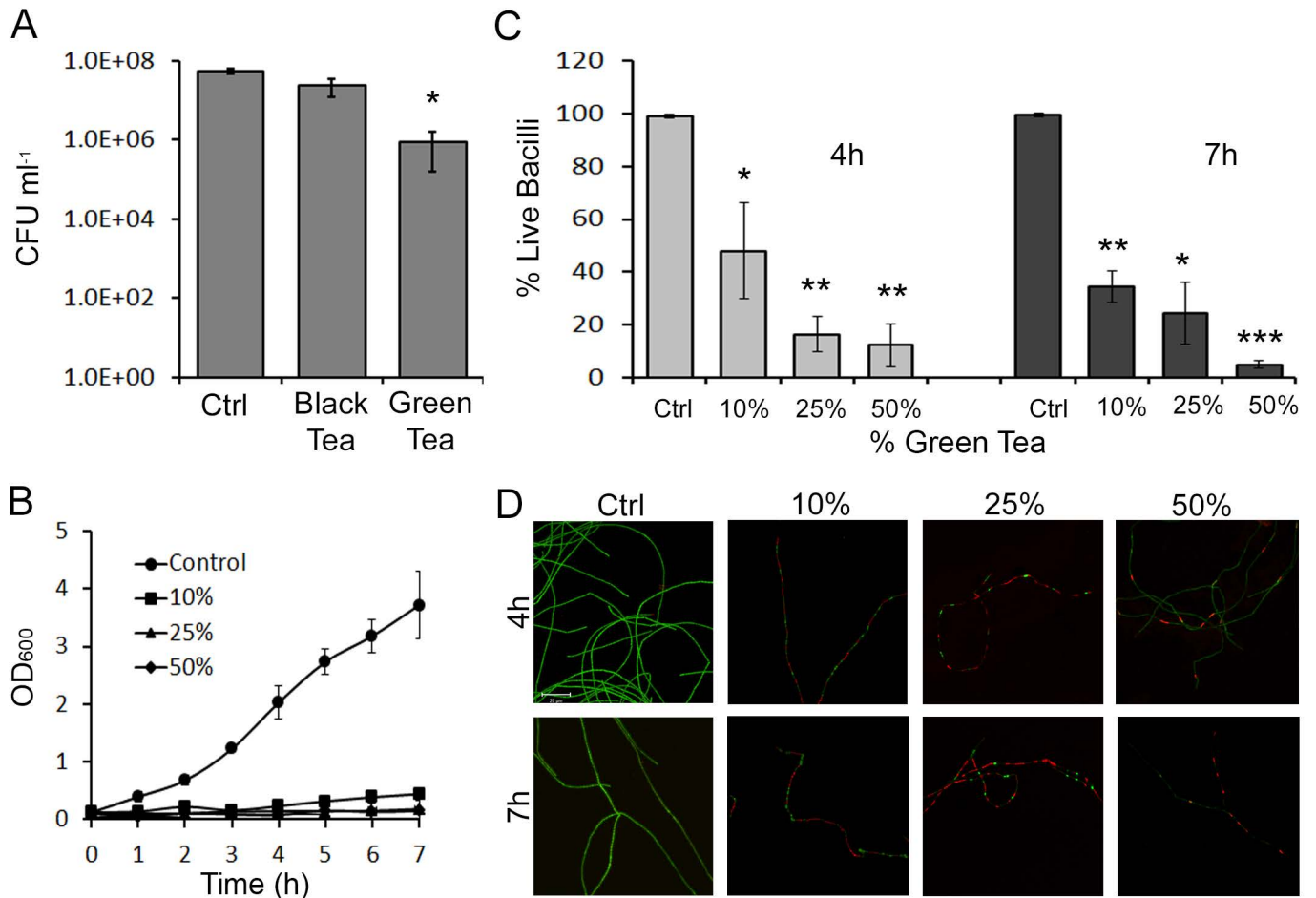


Figure 1

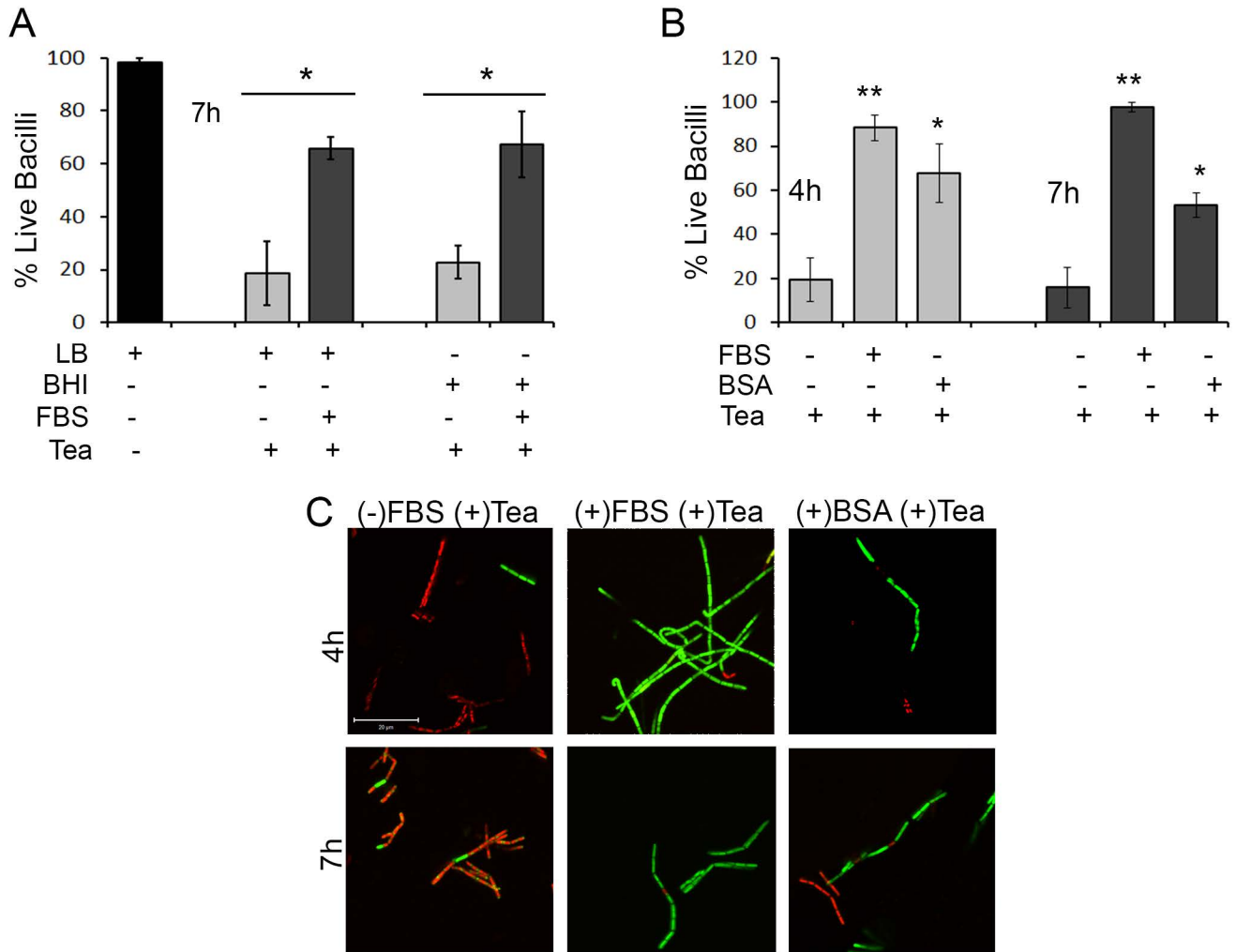


Figure 2

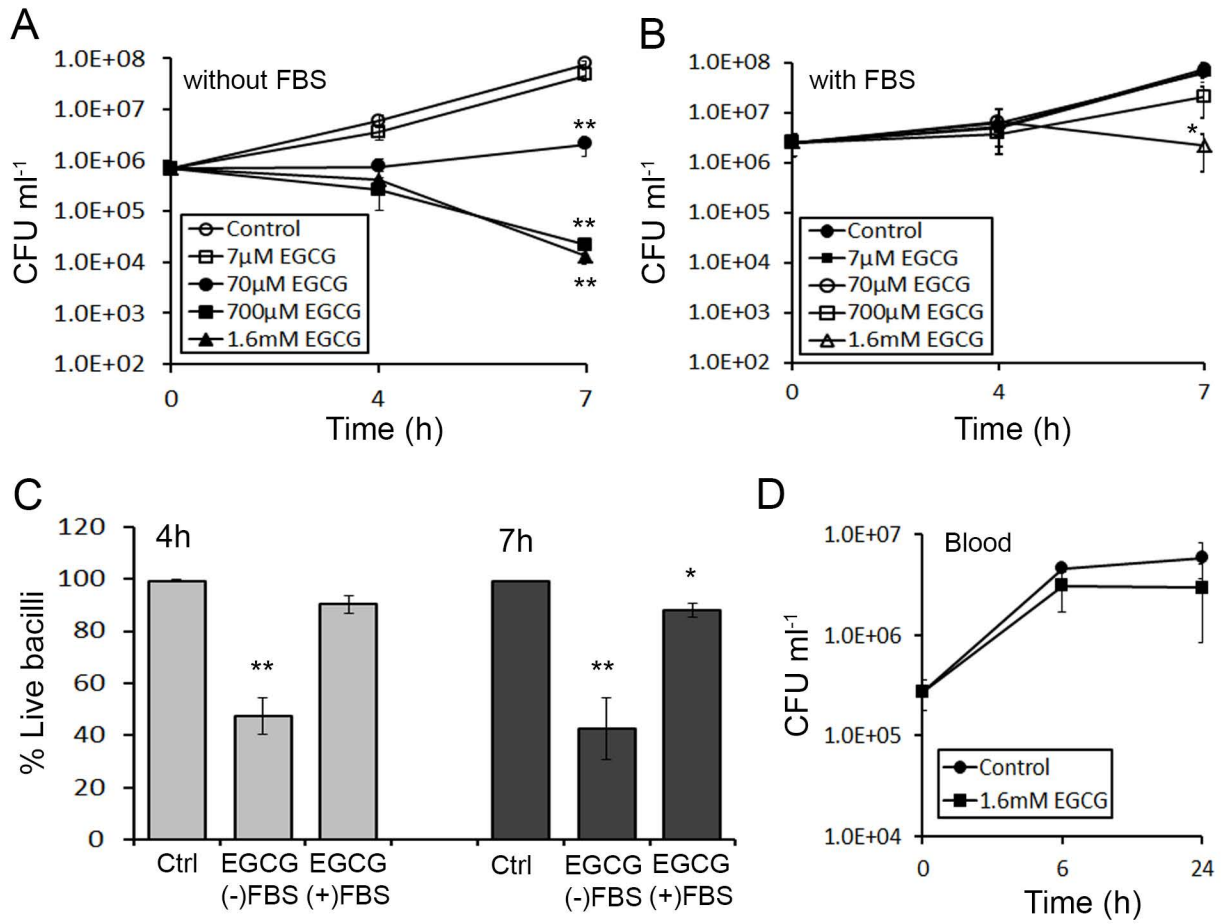


Figure 3

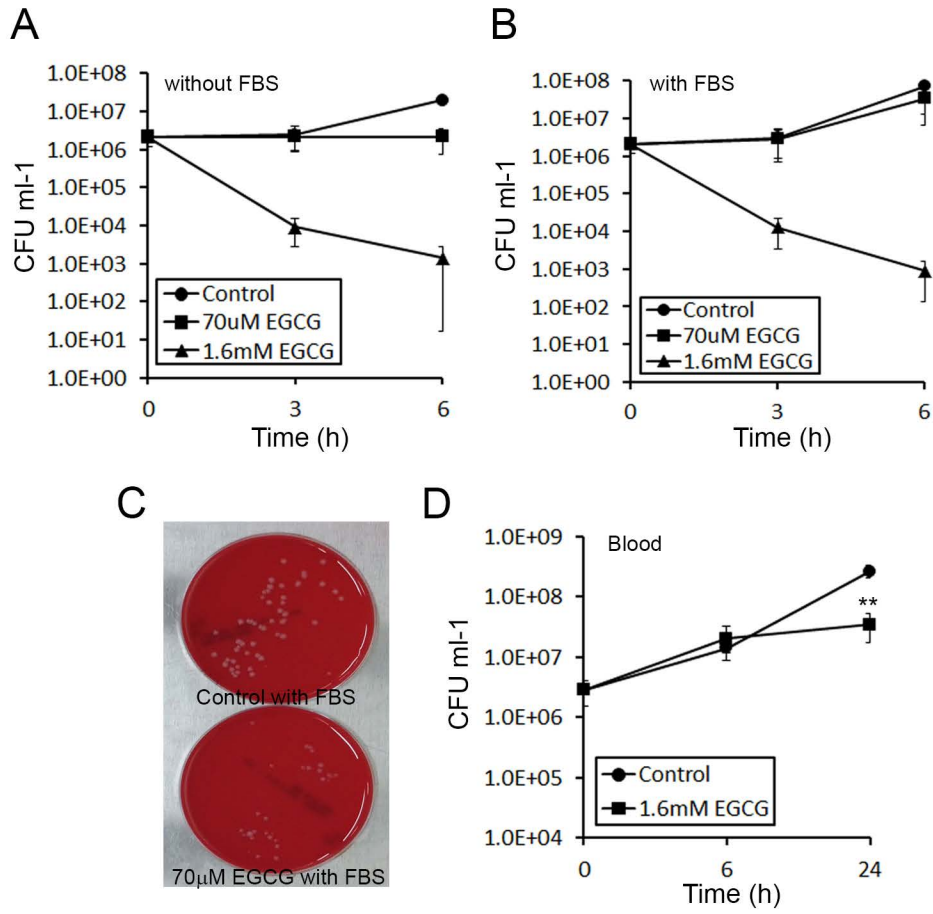
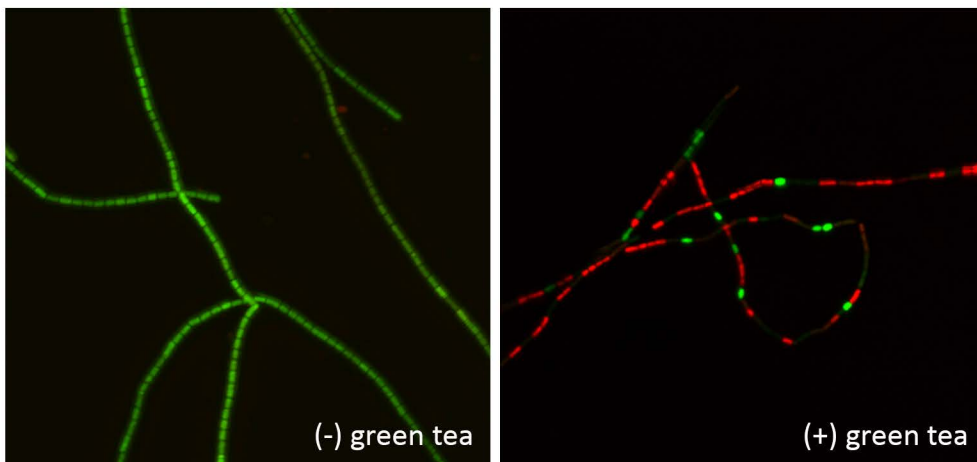


Figure 4



Graphical Abstract