

Synergistic effect of green tea extract and probiotics on the pathogenic bacteria, *Staphylococcus aureus* and *Streptococcus pyogenes*

Ping Su · Anders Henriksson · Christina Nilsson · Hazel Mitchell

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Abstract The aim of this study was to assess the effect of a commercial green tea extract (TEAVIGO™) on the microbial growth of three probiotic strains (*Lactobacillus* and *Bifidobacterium*), as well as three pathogenic bacteria. MIC and co-culture studies were performed. The MICs of the green tea extract against *Staphylococcus aureus* and *Streptococcus pyogenes* ($100 \mu\text{g ml}^{-1}$) were considerably lower than those against the probiotic strains tested ($>800 \mu\text{g ml}^{-1}$) and *Escherichia coli* ($800 \mu\text{g ml}^{-1}$). In co-culture studies, a synergistic effect of the probiotic strains and the green tea extract was observed against both *Staph. aureus* and *Strep. pyogenes*. Green tea extract in combination with probiotics significantly reduced the viable count of both pathogens at 4 h and by 24 h had completely abolished the recovery of viable *Staph. aureus* and *Strep. pyogenes*. These reductions were more significant than the reductions induced by probiotics or green tea extracts used separately. These results demonstrate the potential for combined therapy using the green tea extract plus probiotics on microbial infections caused by *Staph. aureus* and *Strep. pyogenes*. As probiotics and the green tea extract are derived from natural products, treatment with these agents may represent important adjuncts to, or alternatives to, conventional antibiotic therapy.

Keywords Green tea extract · Probiotics · Pathogen · *Staphylococcus aureus* · *Streptococcus pyogenes*

Introduction

Over the last 40–50 years bacteria have become increasingly resistant to commonly used antibiotics, and today we are facing a growing problem in relation to antibiotic resistance, with many infections that were readily cured by antibiotics in the past now being difficult or impossible to treat (Finch 1998). Given this, empirical screening of chemical entities for antimicrobial activity represents an important strategy for the development of novel drugs. Natural products in particular have been a rich source of antimicrobial agents that in general are associated with low levels of toxicity, and in many cases have a fairly broad spectrum of activity (Silver and Bostian 1990).

Tea polyphenols which are constituents of tea extracts have previously been shown to have antibacterial activities against human and animal disease-related bacteria, phytopathogenic bacteria and food-borne bacteria (Sakanaka et al. 2000). For example, Toda et al. (1992) showed that a mixture of tea polyphenols could protect rabbits from experimental infection with *Vibrio cholera*, a finding that led the authors to suggest that patients suffering from cholera could benefit if tea extracts were added to oral rehydration solutions. A commercially available preparation of tea polyphenols, Sunphenon (Taiyo Kagaku Co., Mie), has also been shown to prevent the attachment of a cariogenic *Streptococcus mutans* strain to hydroxyapatite and also to inhibit its glucosyltransferase activity (Otake et al. 1991). The main constituent of tea polyphenols responsible for antibacterial action is epigallocatechin gallate (EGCG) (Yoda et al. 2004). Interestingly, EGCG has also been reported to be a powerful antagonist of human immunodeficiency virus reverse transcriptase, causing 50% inhibition at concentrations of $10\text{--}20 \text{ ng ml}^{-1}$ (Nakane and Ono 1990). TEAVIGO™ is a natural,

P. Su · A. Henriksson · C. Nilsson · H. Mitchell (✉)
School of Biotechnology and Biomolecular Sciences,
The University of New South Wales, Sydney 2052, Australia
e-mail: h.mitchell@unsw.edu.au

caffeine-free, highly purified and refined extract of green tea (*Camellia sinensis*) containing 94% EGCG. Given the high content of EGCG in this natural agent, exploration of its antibacterial activity against human and animal disease-related bacteria is warranted.

Concurrent to the rise in interest in particular chemicals, the specific health effects of selected probiotic strains has become increasingly accepted. Probiotics are live microorganisms, which when administered in adequate amounts confer a health benefit to the host (FAO/WHO). The beneficial effects of probiotics are suggested to be due to a number of factors including modulation of the intestinal bacterial flora, adherence to the mucosa that prevents pathogens from adhering, changes in total enzyme activity in the colon contents, influences on the immune system of the host, and changes in the availability of nutrients, such as short chain fatty acids (Goossens et al. 2003). Indeed studies have shown that probiotics have great potential as adjuncts or alternatives to conventional antibiotic therapy in a range of infectious, inflammatory and allergic diseases (O'Sullivan et al. 2005).

The objectives of this study were to (i) assess the antimicrobial activity of the green tea extract against the pathogenic bacteria *Staph. aureus*, *Strep. pyogenes*, and *E. coli*, (ii) assess the effect of the green tea extract on the growth of representative probiotic strains from the genera lactobacilli and bifidobacteria and (iii) explore the effect of using a combination therapy of the green tea extract plus probiotics against these infectious agents.

Materials and methods

Bacterial strains and growth conditions

Lactobacillus acidophilus LAFTI[®] L10, *Bifidobacterium animalis* ssp. *lactis* LAFTI[®] B94 and *Lactobacillus casei* LAFTI[®] L26 were supplied by DSM Food Specialties, Australia. *Staph. aureus* ATCC 25923, *Strep. pyogenes* ATCC 10389 and *E. coli* ATCC 25922 were obtained from UNSW Microbiology Culture Collection, Sydney, Australia. *Strep. pyogenes* was grown on Horse Blood Agar (HBA, Oxoid, England) plates. All other strains in this study were cultured on Reinforced Clostridial Medium (RCM, Oxoid) agar plates. Inoculated agar plates were incubated at 37°C under anaerobic (AnaeroGen, Oxoid; *L. acidophilus*, *B. animalis* and *L. casei*), microaerophilic (10% CO₂ incubator; *Strep. pyogenes*) or aerobic (*E. coli* and *Staph. aureus*) conditions. Probiotic strains were grown on agar plates for 48 h while the other strains were incubated for 24 h. For culture in liquid media, all strains were grown under appropriate conditions in RCM broth for 24 h. Susceptibility testing was carried out in RCM broth.

To differentiate between bacterial strains following the co-culture experiments, a range of selective agar plates were used. The selective media MGC [MRS (de Man-Rogosa-Sharpe, Oxoid) + bromocresol green + clindamycin], RBP (RCM + aniline blue + propionic acid) and MGV (MRS + bromocresol green + vancomycin) (Su et al. 2007) were used for enumeration of *L. acidophilus* L10, *B. animalis* B94 or *L. casei* L26, respectively. Baird-Parker (Oxoid) agar plates were used for the selective growth of *Staph. aureus*. HBA plates served as the differential medium for *Strep. pyogenes*, as under aerobic, anaerobic and microaerophilic conditions this bacterium is hemolytic on HBA plates. Aerobic conditions were used to detect colonies of *Strep. pyogenes* as under these conditions the background growth of the probiotic strains on HBA plates is reduced.

Assay for inhibition of bacterial growth

The green tea extract tested in this study is commercially available as “TEAVIGO[™]” (DSM Nutritional Products, Australia) containing 94% of EGCG. The green tea extract was dissolved at a concentration of 10 mg ml⁻¹ in milli-Q water, and was sterilized by passing through a 0.45 µm filter. Following filtration, the green tea extract was serially diluted with sterile milli-Q water to appropriate concentrations. The MICs of the green tea extract against bacterial strains were determined by a broth microdilution method using 96-well microtiter plates. Bacterial strains were grown in RCM broth for 24 h. Ten microlitre (approx 5 × 10⁶ c.f.u.) of each bacterial preparation was then inoculated into wells containing 200 µl of RCM broth, supplemented with various concentrations of the green tea extract (0, 25, 50, 100, 200, 400, 800 µg ml⁻¹). All samples were tested in triplicate and were analysed in three independent experiments. After incubation at 37°C for 24 h, all wells were examined macroscopically for bacterial growth, and the lowest concentration of twofold serially diluted green tea extract in which no visible growth had occurred was defined as the MIC.

Bacterial co-cultures in the presence of the green tea extract

To assess the possible synergistic effect of probiotics plus the green tea extract on the growth of pathogenic bacteria, co-cultures of *L. acidophilus* L10, *B. animalis* B94 or *L. casei* L26 together with *Staph. aureus* or *Strep. pyogenes* were performed in 96-well microtiter plates. In brief, bacterial strains were grown in RCM broth under optimal conditions until cell density of each liquid culture reached an optical

density at 600 nm (OD_{600}) ≥ 2.0 . Equal amounts of the individual probiotic and pathogenic isolates in 10 μl were then simultaneously inoculated into microtiter plates. Bacterial combinations were: *Staph. aureus* mono-culture, *Staph. aureus* co-cultured with *L. acidophilus* L10, *Staph. aureus* co-cultured with *B. animalis* B94, *Staph. aureus* co-cultured with *L. casei* L26, *Strep. pyogenes* mono-culture, *Strep. pyogenes* co-cultured with *L. acidophilus* L10, *Strep. pyogenes* co-cultured with *B. animalis* B94, *Strep. pyogenes* co-cultured with *L. casei* L26, *L. acidophilus* L10 mono-culture, *B. animalis* B94 mono-culture and *L. casei* L26 mono-culture. The green tea extract concentrations used in these co-culture experiments were 0, 100, 200 and 400 $\mu\text{g ml}^{-1}$. Samples (100 μl) were collected after 4 h (early stage growth) and 24 h (prolonged growth) of incubation. All samples were serially diluted and analysed for viable counts. Selective agar plates, as described above, were used for the assessment of c.f.u. ml^{-1} .

Results

Minimum inhibitory concentrations of the green tea extract on six bacteria

In order to obtain a universal liquid medium that would support good growth of all the test strains, a number of commercially available media (RCM broth, MRS broth, nutrient broth and Brucella broth, all from Oxoid) were tested. Liquid culture conditions tested included: anaerobic, aerobic using a shaker, and microaerophilic (small bottle with tight lid on shaker—to limit oxygen but ensure good mixing of cells). Inoculum size was also tested, this ranged from a single colony to a full loop inoculum that was inoculated into 5 ml of broth. RCM broth was found to support the growth of each of the test strains, reaching an OD_{600} of more than 2.0 following overnight culture, when optimal culture conditions and inoculum size were used (data not shown). Based on these studies, RCM broth was selected for future assays. In these experiments, the antimicrobial effect of the green tea extract against the six bacterial strains was assessed in RCM broth containing 0, 25, 50, 100, 200, 400 and 800 $\mu\text{g ml}^{-1}$ of the green tea

extract under anaerobic conditions. Visible growth of bacteria was determined and the lowest concentration showing no growth was defined as the MIC. As shown in Table 1, the six tested strains showed different susceptibilities to the green tea extract. As compared to the MICs of the green tea extract against *Staph. aureus* and *Strep. pyogenes*, the MICs of the green tea extract against the three probiotic strains and *E. coli* were considerably higher (Table 1). The MICs of the green tea extract against *Staph. aureus* and *Strep. pyogenes* were also tested under aerobic and microaerophilic culture conditions and similar results were obtained (data not shown). Thus, based on these results the subsequent co-culture experiments were carried out under anaerobic conditions.

Synergistic effect of the green tea extract and probiotic strains on the growth of bacterial pathogens

In order to determine if a combination of probiotics plus the green tea extract would have a synergistic effect on the growth of certain pathogens e.g. *Staph. aureus* and *Strep. pyogenes*, we further performed co-culture studies using *L. acidophilus* L10, *B. animalis* B94 or *L. casei* L26 with *Staph. aureus* and *Strep. pyogenes* in the absence or presence of the green tea extract.

Growth of the probiotic strains *L. acidophilus* L10, *B. animalis* B94 or *L. casei* L26 was not affected by the presence of the green tea extract or by the presence of *Staph. aureus* and *Strep. pyogenes*. The viable counts of the probiotic strains remained unchanged in green tea extract concentrations ranging from 0 to 400 $\mu\text{g ml}^{-1}$. The colony count of the co-cultured probiotic strains had similar values to that of the mono-cultured probiotic strains alone, at both 4 and 24 h (data not shown).

In contrast to the probiotic strains, the green tea extract led to a significant decrease in c.f.u. of both *Staph. aureus* and *Strep. pyogenes* and this was observed at an early stage (4 h). This decrease was dependent on the concentration of the green tea extract (Figs. 1 and 2). Inhibition of these pathogens by green tea extract was further increased in the presence of the co-cultured probiotics *L. acidophilus* L10, *B. animalis* B94 or *L. casei* L26. The viable count of *Staph.*

Table 1 Comparison of MICs of green tea extract TEAVIGO™ against three strains of pathogenic bacteria and three probiotic strains

Pathogenic bacteria		Probiotic strains	
Test strain	MIC ($\mu\text{g ml}^{-1}$)	Test strain	MIC ($\mu\text{g ml}^{-1}$)
<i>Staph. aureus</i> ATCC 25923	100	<i>L. acidophilus</i> L10	>800
<i>Strep. pyogenes</i> ATCC 10389	100	<i>B. animalis</i> B94	>800
<i>E. coli</i> ATCC 25922	800	<i>L. casei</i> L26	>800

All experiments were performed in triplicate in three separate experiments

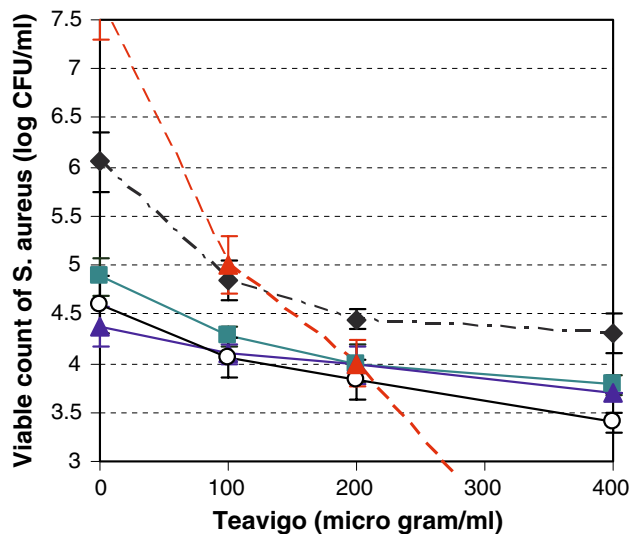


Fig. 1 The effect of the green tea extract concentration in combination with different probiotic strains on the viability of *Staph. aureus*. The graph shows the number of c.f.u. recovered from selective plates for *Staph. aureus*. · — ◆ — · : *Staph. aureus* mono-culture at 4 h; —■— : *Staph. aureus* co-cultured with *L. acidophilus* L10 at 4 h; —▲— : *Staph. aureus* co-cultured with *B. animalis* B94 at 4 h; —○— : *Staph. aureus* co-cultured with *L. casei* L26 at 4 h; - - - ▲ - - - : *Staph. aureus* mono-culture at 24 h. When co-cultured with each of the test probiotics, *Staph. aureus* dropped below the detection level after 24 h incubation, thus are not depicted on the graph. The results are the mean of three independent experiments, vertical bars represent standard deviations

aureus was further reduced to 2- to 15-fold and the viability of *Strep. pyogenes* was reduced to 3- to 30-fold compared to green tea extract alone at all concentration tested (Figs. 1 and 2). A synergistic effect was observed for all tested combinations of probiotic strains and the green tea extract with significantly reduced growth of both *Staph. aureus* and *Strep. pyogenes* was observed. Of the three probiotics tested, *L. casei* L26 gave the greatest effect when used in combination with the green tea extract (Figs. 1 and 2).

Examination of the effect of the dosage of the green tea extract alone showed that after 24 h incubation, the inhibitory effect became more obvious. In RCM broth without the green tea extract, cells grew to approximately 10^8 c.f.u. ml⁻¹ (Figs. 1 and 2). However, as the concentration of the green tea extract in the broth increased, the c.f.u. significantly decreased. At a concentration of 400 μ g ml⁻¹ of the green tea extract, *Staph. aureus* was totally eliminated and after 24 h incubation the concentration of *Strep. pyogenes* dropped to 9×10^2 c.f.u. ml⁻¹. The growth inhibitory effect of bacterial co-cultures was also more significant at 24 h. Even without the green tea extract, the growth of *Staph. aureus* and *Strep. pyogenes* was completely inhibited by *L. acidophilus* L10, *B. animalis* B94 or *L. casei* L26 after 24 h incubation. Incubation under microaerophilic and

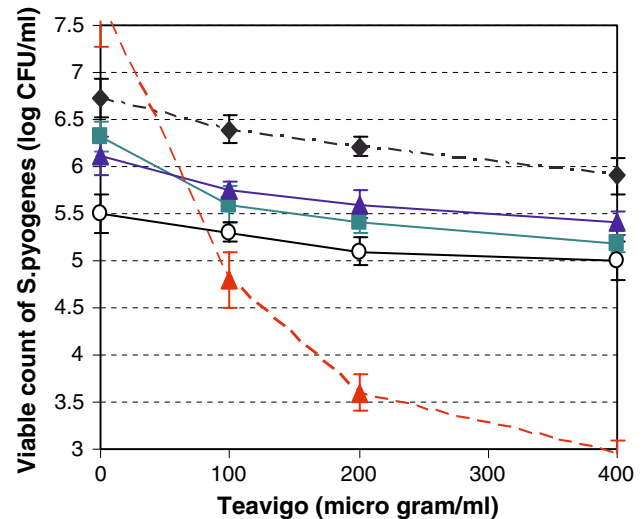


Fig. 2 The effect of the green tea extract concentration in combination with different probiotic strains on the viability of *Strep. pyogenes*. The graph shows the number of c.f.u. recovered from selective plates for *Strep. pyogenes*. · — ◆ — · : *Strep. pyogenes* mono-culture at 4 h; —■— : *Strep. pyogenes* co-cultured with *L. acidophilus* L10 at 4 h; —▲— : *Strep. pyogenes* co-cultured with *B. animalis* B94 at 4 h; —○— : *Strep. pyogenes* co-cultured with *L. casei* L26 at 4 h; - - - ▲ - - - : *Strep. pyogenes* mono-culture at 24 h. When co-cultured with each of the test probiotics, *Strep. pyogenes* dropped below the detection level after 24 h incubation, thus are not depicted on the graph. The results are the mean of three independent experiments, vertical bars represent standard deviations

aerobic conditions gave similar results for *L. acidophilus* L10 and *L. casei* L26. However, *B. animalis* B94 grew less prolifically under these conditions and thus the inhibitory effect of this strain was less obvious under microaerophilic and aerobic conditions. Nevertheless, the synergistic effect of the green tea extract and *B. animalis* B94 strengthened the inhibitory effect on both *Staph. aureus* and *Strep. pyogenes* under these conditions (data not shown).

Discussion

In the initial stages of this study, we investigated the ability of green tea extract TEAVIGO™ to inhibit the growth of selected pathogens. These studies showed that the green tea extract exerts an inhibitory effect on the growth of two major human pathogens *Staph. aureus* and *Strep. pyogenes*. In contrast, *E. coli* was unaffected by the presence of the green tea extract. In addition, we showed that the probiotic strains *L. acidophilus* L10, *B. animalis* B94 and *L. casei* L26 were also unaffected by the green tea extract at the concentrations used in the study. These findings are in contrast to a number of previous studies, which have suggested that Gram-positive bacteria are more sensitive to EGCG than Gram-negative bacteria (Yoda et al. 2004).

More recently however Taguri et al. (2006) have reported that the antimicrobial potency of polyphenols was dependent upon the bacterial species and was not correlated with the Gram stain. In their study, which investigated the MIC of EGCG against 26 bacterial species, the authors showed that while the antibacterial activity of polyphenols against *Aeromonas hydrophila*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* was reasonably strong, activity against 11 species of the Enterobacteriaceae was comparatively weak and that against six species of aerobic bacterial plant pathogens was moderate (Taguri et al. 2006). Taguri et al's conclusion that the antimicrobial potency of polyphenols is dependent upon bacterial species is consistent with our findings, which showed that, while the green tea extract was active against two Gram-positive bacteria, *Staph. aureus* and *Strep. pyogenes*, it did not affect the activity of *E. coli* or the three Gram-positive probiotic strains *L. acidophilus* L10, *B. animalis* B94 and *L. casei* L26, at the concentrations tested here. Moreover, Goto et al. (1999) who evaluated the effects of tea catechins on faecal contents and metabolites of elderly residents in a long-term care facility, demonstrated that consumption of green tea selectively promoted the growth of *Bifidobacterium* and *Lactobacillus* in the gut wall. Known that in our study green tea extract could significantly decrease the survival of *Staph. aureus* and *Strep. pyogenes*, but did not affect the test probiotic strains, it is possible that the effect seen by Goto et al. in vivo might be due to the suppression of other bacteria in the intestinal tract, which would provide a niche for the increased growth of the probiotics.

Given that addition of the green tea extract to the culture medium did not affect the growth of *L. acidophilus* L10, *B. animalis* B94 or *L. casei* L26, the possibility that these probiotic strains could be used in combination with the green tea extract as a therapeutic to reduce the growth of microbial pathogens in human infections was raised. In order to determine if the two components would have a synergistic effect, we performed in vitro co-cultures of the probiotics and *Staph. aureus* or *Strep. pyogenes*, the two species that had shown reduced viability at increasing the green tea extract concentrations. These studies showed that, starting from an early stage of growth (4 h), the simultaneous presence of *L. acidophilus* L10, *B. animalis* B94 or *L. casei* L26 and the green tea extract reduced the number of c.f.u. recovered from both pathogens, and that after 24 h of growth this effect was even more pronounced. Comparison of these results to those obtained when either the green tea extract or probiotics alone was present, demonstrated that the reduction in c.f.u. was significantly greater in the co-cultures containing both the green tea extract and probiotic, thus suggesting a synergistic effect.

A balanced composition of the microbial flora in the gastro-intestinal tract has been reported to be important for

the well-being of an individual (Sullivan and Nord 2005). Disturbances due to, for example, the introduction of pathogenic micro-organisms or the use of antibiotics may severely impact upon this balance. Recent studies would suggest that probiotics can restore the microbial balance in the host and that they may be promising candidates to be included in both prophylactic and therapeutic agents (Mack and Lebel 2004). However, the beneficial effects of probiotics may not always be sufficient, thus the identification of alternative agents that may confer an additive effect to their action is likely to be of great value. It is possible that the synergistic effect of the green tea extract and probiotics observed in the present study may be due to a symbiotic consequence as well as additional mechanisms offered by the green tea extract.

In conclusion, the present study has shown that a synergistic effect exists between the green tea extract and the probiotic strains *L. acidophilus* L10, *B. animalis* B94 and *L. casei* L26 in relation to the inhibition of in vitro growth of the important human pathogens, *Staph. aureus* and *Strep. pyogenes*. These findings not only raise the possibility that the green tea extract in combination with probiotics may be useful for the treatment of specific *Staph. aureus* and *Strep. pyogenes* infections, but also that they may be beneficial for the treatment of other infectious conditions. Further in vivo studies, investigating the synergistic effect of the green tea extract and probiotics against a range of bacterial pathogens are precedence. Such investigations could reveal novel treatment strategies for infectious diseases that today are being increasingly more difficult to treat due to the global spread of antibiotic resistance.

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