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Inhibitory Effects of Commercial and Enriched Green Tea Extracts on the Growth of *Brochothrix thermosphacta*, *Pseudomonas putida* and *Escherichia coli*

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Abstract

The major catechin found in green tea, called epigallocatechin gallate (EGCG), have been reported to have antimicrobial properties. In this study, we examined *in vitro* the antimicrobial effects of a commercial green tea extract sold in a capsule form, and two prepared green tea extracts enriched in catechins against *Brochothrix thermosphacta*, *Pseudomonsas putida* and *Escherichia coli* which have been associated with meat spoilage. The antimicrobial activity of the different tea extracts was evaluated by Spot-On-Lawn and Well Diffusion assays and the Minimum Inhibitory Concentration (MIC) was also determined in Brain Heart Infusion broth. The three methods used showed an inhibition of *Brochothrix thermosphacta*, whereas the inhibition of *Pseudomonas putida* and *Escherichia coli* was only detected with the MIC assay. The determination of the MIC in broth culture appeared to be the most reliable method to determine the inhibitory activity of catechin compounds.

Keywords: catechins, green tea, inhibition, meat, antimicrobial

1. Introduction

Green tea is obtained from *Camellia sinensis* leaves that are steamed and dried. These processes result in an inactivation of the polyphenol oxidase, and thus, contributes to maintain the polyphenols in their monomeric forms (Higdon & Frei, 2003). Polyphenols are the most important components of tea leaves and catechins are the principal group representing 30 to 40% of the dry weight. Amongst catechins, (-)epigallocatechin gallate (EGCG) remains the most abundant and the most widely studied (Almajano, Carbo, Jiménez, & Gordon, 2008). Recently, green tea and EGCG have gained the attention of the scientific community because of their health benefits as antioxidants, anti-angiogenics (growth prevention of blood vessel tumours) and anti-mutagenic agents (Cabrera, Artacho, & Giménez, 2006). Green tea has also demonstrated anti-diabetic properties with respect to insulin resistance in animal models, as well as antibacterial, anti-HIV, anti-aging and anti-inflammatory activities (Zaveri, 2006).

The polyphenolic components of tea have been found to be extremely effective against various strains of foodborne bacteria including pathogenic strains such as *Clostridium perfringens*, *Vibrio parahaemolyticus*, *Vibrio metchnikovii*, *Staphylococcus aureus*, *Bacillus cereus*, *Plesiomonas shigelloides* and *Aeromonas sobria* (Hara, 2001). Green tea has also antimicrobial effects against *Helicobacter pylori*, the organism involved in gastric, peptic and duodenal ulcer diseases (Dufresne & Farnworth, 2001). In this context, we tested the antimicrobial activity of various green tea extracts against *Brochothrix thermosphacta*, *Pseudomonsas putida* and *Escherichia coli,* which have been associated with meat spoilage, to evaluate their potential as a natural source of antimicrobial ingredients to be used in food or feed.

2. Materials and Methods

2.1 Preparation of the Tea Extracts

Commercial green tea capsules were purchased from a local drug store (Pharmacie des Vosges, Nice, France).

Sencha green tea leaves (Trans-Herbe Inc., St-Bruno, Quebec, Canada) were used to prepare different green tea extracts to be tested as antimicrobial solutions. Two green tea extracts enriched in catechins were prepared in our laboratory according to the procedure patented by Bazinet, Labbé and Tremblay (2009). Thirty-five grams of dried and non organic Sencha leaves were brewed twice in 1 L of water. The first brewing was carried out at 30°C ± 2°C for 10 min. The infusion obtained was filtered to separate the leaves from the liquid phase and was named "extract 1-30°C". The tea leaves collected after the first infusion were brewed one more time in 1 L of water at 80°C ± 2°C for 1 h. This new infusion was filtered again to remove the leaves and the resulting solution was named "extract 2-80°C". Both extracts were frozen at -20°C for 48 h, and lyophilised at 25°C for 72 h (Virtis 509851, Gardinier, NY). To test the antimicrobial activity of the different green tea extracts (commercial and catechins enriched ones), the lyophilised powders were solubilised at a concentration of 50 mg mL^{-1} in distilled water and were centrifuged (RC3C, Sorvall Instrument, Dupont, Wilmington, DE, US) at 960 × g for 10 min at room temperature to remove any residue. Samples were then filtered through a 0.45 µm filter (Sterile Acrodisc® Syringe Filters, Pall, Ville St-Laurent, QC, Canada).

2.2 Bacterial Strains and Culture Conditions

Brochothrix thermosphacta ATCC 11509, *Escherichia coli* ATCC 25922 and *Pseudomonas putida* CRDAV 371 were obtained from the culture collection of the Food Research and Development Centre of Agriculture and Agri-Food Canada in St-Hyacinthe. Stock cultures were stored at -80°C in Brain Heart Infusion broth (BHI; Becton, Dickinson and Company, Mississauga, ON, Canada) supplemented with 20% (v/v) glycerol (FisherBiotech, Fairlawn, NJ, USA). Strains were individually subcultured (1% v/v) in BHI broth for a minimum of two and a maximum of seven consecutive days before being used in the experiments. Cultures were incubated for 24 h at 22°C for *B. thermosphacta* and *P. putida*, and at 37°C for *E. coli*.

2.3 Evaluation of Antimicrobial Activities of Green Tea Extracts

Three different methods were used to characterize the inhibitory activity of green tea on bacterial growth. For the Spot-On-Lawn assay (Gratia, 1946; cited by Tagg, Dajani, & Wannamaker, 1976), 15 mL of BHI soft agar (7.5% agar) was melted and inoculated (1% v/v) with an overnight culture of one of the target microorganism. The inoculated soft agar was then mixed and poured onto a pre-solidified BHI agar plate. A 20 µL aliquot of the solution prepared from the different tea extracts was spotted onto the inoculated soft agar and dried under a biosafety cabinet. The diameter of the inhibition zone was then measured precisely with a caliper (Mitutoyo Corporation Ltd., Mississauga, ON, Canada) after 18 h of incubation at the same growth temperature as described above for each microorganism tested.

The second method used was the Well Diffusion assay previously described by Schillinger and Lücke (1989). First, 25 mL BHI soft agar (7.5%) was melted and inoculated at 1% with an overnight culture of the organism to be tested. The inoculated soft agar was mixed, poured into a sterile petri dish and stored at 4°C for 2 h for solidification. Wells of 6 mm diameter were made using the wide opening at the top of a 10 mL disposable sterile pipette. An 80 µL aliquot of the tea solutions was placed in each well. Inhibition diameters were measured after 18 h of incubation as described above. Zones were measured from the edge of the well to the end of inhibition zone.

At last, the Minimum Inhibitory Concentrations (MIC) of the green tea solutions were determined using the microplate assay previously described by Mota-Meira, Lapointe, Lacroix and Lavoie (2000). A volume of 125 µL of sterile BHI broth was added to each well of a sterile 96-well U-bottom plate (BrandPlates® Pure GradeTMS, Brandtech, Essex, CT, USA). The same volume of inhibitory solution (125 µL) was added to the first well and serial dilutions (1:2) of each green tea solutions studied were made. The optical density ($OD_{600\ nm}$) of the overnight cultures in BHI broth was adjusted to 0.1 with fresh BHI broth (Benchmark Microplate Reader, Bio-Rad, Hercules, CA, USA) which corresponds to a 0.5 McFarland standard (1-2 x 10^8 CFU mL^{-1}) and a volume of 50 µl was added to each well. The microplates were incubated for 18 h at 22°C for *B. thermosphacta* and *P. putida* and 37°C for *E. coli* and bacteria growth was evaluated by absorbance at 655 nm which is away from absorbing wavelengths of catechins compounds in BHI broth (Kheadr, Bernoussi, Lacroix & Fliss, 2004). The precision of the microplate reader is 0.002 OD. BHI broth without cell was used as a negative control and BHI broth without antimicrobial compound as a positive control. The MIC was determined as the lowest concentration of tea extract required for a complete growth inhibition of the target microorganism ($OD_{655\ nm}$ of well with cells and antimicrobial equal to $OD_{655\ nm}$ of the negative control).

2.4 Analysis of Catechins Content of Green Tea Extracts by RP-HPLC

The composition of the extracts was characterized by reverse phase high pressure liquid chromotography (RP-HPLC) as previously described by Labbé, Têtu, Trudel and Bazinet (2008). Each final solution of green tea

was filtered through a 0.2 µm filter (Acrodisc® LC13 PVDF, Gelman Laboratory, Ann Arbor, MI, USA) and diluted (1:10) with HPLC grade water. Standard curves were calculated from a mix of catechins and caffeine compounds (six concentrations ranging from 0 to 50 mg mL^{-1}): (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin gallate (EGCG), (-)-gallocatechin gallate (GCG) and caffeine. Standards were purchased from the Sigma-Aldrich Company (St-Louis, MO, USA) and retention time was determined for each individual compound by injection of known concentrations. The RP-HPLC method was based on the National Institute of Standards and Technology method (Dalluge, Nelson, Brown Thomas & Sander, 1998) and experiments were performed under the same conditions as Labbé, Têtu, Trudel and Bazinet (2008). Detection of analytes was accomplished by UV detection at 210 nm (WatersTM 486 Tunable Absorbance Detector, Waters Inc, Lachine, QC, Canada). The temperature of the column was maintained at 25°C during the analysis and the mobile phases constituted of an aqueous solution of 0.05% trifluoroacetic acid (TFA, HPLC grade; JT Baker, Phillipsburg, NJ, USA) for phase A and 0.05% TFA in acetonitrile (HPLC grade, EMD Chemicals Inc., Gibbstown, NJ, USA) for phase B. Both solutions were filtered through 0.2 µm nylon filters before use (Mendel Scientific Compagnie, Guelph, ON, Canada).

2.5 Statistical Analysis

Statistical analysis was performed using Statview 5.0 software (SAS Institute). Three biological repetitions (n = 3) were analyzed in triplicate for all experiments except for the MICs which were done in duplicate. All numeric values were reported as means ± standard deviation except for the MIC where results were reported as median. Homogeneity of the variance between groups was assessed using the Bartlett test. The effects of the different experimental treatments were evaluated using a one-way ANOVA, followed by a Student-Newman-Keuls test for multiple mean comparisons except when variance homogeneity was not observed. Hence, EGC and ECG contents were analyzed by the Kruskall and Wallis non-parametric test and the means were compared using the Mann-Whitney U-test. All tests were performed with a 5% significance level.

3. Results and Discussion

The antimicrobial activities of two different aqueous green tea extracts enriched in catechins have been studied and compared to those of a commercial tea capsule. Using the Spot-On-Lawn assay, the diameter of the inhibition zone of the commercial capsule was evaluated at 11.87 ± 1.42 mm for *B. thermosphacta*, whereas it had no effect against *P. putida* and *E. coli*. None of the two catechins enriched extracts demonstrated an inhibitory activity against the three spoilage bacteria tested with this technique.

Surprisingly, the commercial capsule showed no inhibitory activity against *B. thermosphacta* or against the two other bacteria tested, using the Well Diffusion assay method. Furthermore, contrary to what was observed with the Spot-On-Lawn technique, the two enriched extracts demonstrated inhibitory effects against *B. thermosphacta.* Zones of inhibition observed with the Well Diffusion assay were the largest (P = 0.04) with the 2-80°C extract (6.67 ± 0.71 mm), compared to the 1-30°C extract (4.29 ± 1.19 mm). *P. putida* and *E. coli* did not seem to be sensitive to any of the three green tea solutions regardless of the agar assay used. The different results obtained with the Spot-On-Lawn and the Well Diffusion assays for the commercial capsule and the enriched extracts against *B. thermosphacta* may indicate that there are differences in catechin diffusion that depend on the antimicrobial technique used**.** The main difference between the two methods is that in the Spot-On-Lawn assay, the solution containing the antimicrobial compounds is in direct contact with the cells embedded in the soft agar. In the Well Diffusion assay, the solution must first diffuse through the agar to have an effect (Lewus & Montville, 1991). The fact that no inhibition was observed for the commercial capsule extract against *B. thermosphacta* with the Well Diffusion assay suggests that the antibacterial compounds do not diffuse readily through the agar to produce a distinctive zone of inhibition. Furthermore, the area of diffusion is more important in the Well Diffusion assay than for the Spot-On-Lawn method and the active compounds may be concentrating during a rather slow diffusion process. Optimal growth temperature was different depending on the microorganisms tested and might also have influenced diffusion.

Table 1 presents the Minimum Inhibitory Concentrations of the commercial capsule and the two other extracts against *B. thermosphacta*, *P. putida* and *E. coli*, evaluated in BHI broth. In accordance with the previous results obtained with the Spot-On-Lawn and the Well Diffusion assays, *B. thermosphacta* was the most sensitive strain. MICs of 4.69, 1.56 and 3.13 mg ml^{-1} were observed for the commercial capsule, the 1-30°C and the 2-80°C extracts, respectively. Contrary to the results obtained for the Spot-On-Lawn and the Well Diffusion assay on solid agar, all green tea extracts inhibited *P. putida* and *E. coli* growth when the MICs were determined by growth in BHI broth.

Table 1. MICs[a] (mg of powder mL^{-1}) of the various green tea solutions against *B. thermosphacta*, *P. putida* and *E. coli*

Fraction	*B. thermosphacta*	*P. putida*	*E. coli*
Capsule	4.69 (3.13 - 6.25)	18.75 (12.50 - 25)	6.25
1-30°C	1.56 (0.78 - 3.13)	3.13 (1.56 - 6.25)	25
2-80°C	3.13 (1.56 - 12.50)	25 (3.13 - > 50)	> 50 (25 - > 50)

[a] The MICs were performed using serial dilutions (1:2). Data obtained from at least three independent repetitions were done in duplicate for each strain and are expressed as the median and the range of values obtained are in parentheses (discrete value; data presented according to Mota-Meira, Lapointe, Lacroix and Lavoie, 2000). Result "> 50" indicates that no inhibition was observed even at the highest concentration.

The 1-30°C extract was the most effective solution to inhibit *P. putida* with a MIC of 3.13 mg mL^{-1} compared to 18.75 and 25 mg mL^{-1} for the commercial capsule and the 2-80°C extract, respectively. The most effective solution against *E. coli* was the commercial capsule with a MIC of 6.25 mg mL^{-1} compared to 25.0 mg mL^{-1} for 1-30°C extract; no inhibition was observed with the 2-80°C extract at the concentrations tested. Overall, *B. thermosphacta* was the most sensitive strain tested. The larger spectrum of inhibition with the MIC assay was probably due to the fact that the cells were in direct contact with the active compounds in liquid medium, as no diffusion in agar was involved. Furthermore, other authors have found that the broth assay were more efficient than agar assays for a large number of antibiotics (Baker, Stocker, Culver, & Thornsberry, 1991). According to Hara (2001), the EGC, EGCG and ECG catechins seem to be the most potent antibacterial agents and the activity of catechins alone is reported to be modest (Taylor, Hamilton-Miller, & Stapleton, 2005). Hence, the observed results may be considered as a synergistic effect between the different compounds found in green tea.

In order to explain the different antimicrobial effects, the catechin composition was evaluated for the different solutions obtained from the tea extracts and the commercial capsule (Table 2). The extracts 1-30°C and 2-80°C were not different from each other in their EGC, caffeine and epicatechin (EC) content ($P > 0.05$). The concentration of EGC was higher in both catechins enriched extracts (1-30°C and 2-80°C), compared to the commercial capsule solution ($P = 0.02$). The concentration of EC was significantly lower ($P = 0.007$) for the commercial capsule compared to the two enriched green tea leaves extracts. In 1-30°C extract, the concentration of EGCG and ECG was significantly lower than one of the two other extracts ($P = 0.003$). The concentration of caffeine, EGCG and ECG in 2-80°C extract and the commercial capsule were not significantly different ($P > 0.05$; Table 2). The three extracts differed significantly in their concentrations of gallocatechin gallate (GCG; $P < 0.001$). The concentration of GCG was below the HPLC detection limits for the 1-30°C extract, but was detected at 0.42 ± 0.09 and 3.53 ± 0.25 mg mL^{-1} for 2-80°C extract and the commercial capsule, respectively. Our catechins composition results for the 2-80°C extract were similar to those reported by Bazinet, Araya-Farias, Doyen, Trudel and Têtu (2010) who obtained their extract using a similar protocol than in our study. It is also known that the process used to brew the tea, the cultivar, the culture conditions and the time of harvest are important factors that may influence the final catechin content of the brewed tea (Hara, 2001). This also explains why the commercial capsule was different from our extracts (Table 2).

Overall, the main differences between the commercial capsule and the enriched extracts were the EGC, EC and GCG content (Table 2). The EC catechin has not been reported in the literature to have an antibacterial effect on the bacteria used in this study. The GCG has been reported to be effective against pathogenic sporulating bacteria such as *Clostridium botulinum* and *Bacillus cereus* (Friedman, Henika, Levin, Mandrell & Kozukue, 2006; Hara, 2001). The highest GCG content in the commercial capsule could explain why *E. coli* was inhibited more efficiently by the commercial capsule in the MIC assay. However, non-catechin compounds may also account for the observed inhibition. Taguri, Tanaka and Kouno (2004) have also tested EGC and EGCG pure compounds in aqueous solution against *E. coli* ATCC 25922. They mixed catechin solutions with 18 mL of Mueller Hinton agar inoculated with *E. coli* at a concentration of 9.4×10^3 CFU mL^{-1}. These authors found that *E. coli* was fully inhibited by 800 μg ml^{-1} of EGC or 400 μg mL^{-1} of EGCG. In our study, *E. coli* was more sensitive to the 1-30°C extract and the capsule than to the 2-80°C extract. The observed inhibition appears to be multifactorial and influenced by both the composition and the method used to access the antimicrobial activity.

Table 2. Catechin composition and proportion for the commercial capsules and the two green tea extracts

Composition*		Capsule	Extract 1-30°C	Extract 2-80°C
EGC	mg mL^{-1}§	2.29 ± 0.27^{b}	4.42 ± 0.09^{a}	4.94 ± 0.88^{a}
	%	13.81	38.82	29.87
Caffeine	mg mL^{-1}	3.70 ± 0.36^{a}	2.87 ± 0.26^{a}	3.55 ± 0.60^{a}
	%	22.51	25.15	21.46
EC	mg mL^{-1}	0.67 ± 0.11^{b}	1.08 ± 0.07^{a}	1.18 ± 0.19^{a}
	%	4.02	9.49	7.15
EGCG	mg mL^{-1}	4.94 ± 0.29^{a}	2.46 ± 0.60^{b}	5.20 ± 0.86^{a}
	%	29.82	21.61	31.41
GCG	mg mL^{-1}	3.53 ± 0.25^{a}	BDL^{b}	0.42 ± 0.09^{b}
	%	21.31	BDL$^{\#}$	2.55
ECG	mg mL^{-1}	1.41 ± 0.01^{a}	0.56 ± 0.16^{b}	1.25 ± 0.22^{a}
	%	8.53	4.94	7.56
Total	mg mL^{-1}	16.57 ± 1.28^{a}	11.40 ± 0.70^{b}	16.55 ± 2.80^{a}
	%	100	100	100

* EGC = epigallocatechin; EC = epicatechin; EGCG = epigallocatechin gallate; GCG = gallocatechin gallate; ECG = epicatechin gallate.

BDL = Below Detection Level.

§ Catechin concentrations are means ± standard deviation (n = 3). Means with the same letter in the same line do not differ significantly (P > 0.05). Percentage represents the quantity of identified compound over the overall compounds analysed.

Finally, green tea extracts seem to be more effective on gram positive (*B. thermosphacta*) than gram negative (*P. putida* and *E. coli*) bacteria, as already reported in the literature (Chiu & Lai, 2010; Negi, Jayaprakasha & Jena, 2003; Yilmaz, 2006). The gram negative resistance is most likely due to their outer membrane composed of lipopolysaccharide, which is more resistant to chemical compounds (Chiu & Lai, 2010), while gram positive bacteria are most sensitive to physical treatments. Furthermore, Si et al. (2006) have reported that green tea extracts block cell division in *Bacilus cereus* and this may be true for other gram positive bacteria. Hence, polyphenols extracted from green tea have interesting antimicrobial properties worth investigating in food systems. More recently, feed supplementation with natural sources of polyphenols in rabbits and pigs was effective in improving the microbial quality of the fresh meat stored under aerobic conditions (Fortier, Saucier & Guay, *in press;* Soultos, Tzikas, Christaki, Papageorgiou, & Steris, 2009).

4. Conclusion

B. thermosphacta was the most sensitive strain to green tea extracts amongst the three tested. In this study, we demonstrated that the classical method used to detect antimicrobial activities influences the antimicrobial activity observed. Because the target organism is in direct contact with the active ingredients, without having to go through diffusion in a solid medium (in agar here), the determination of the MIC in broth culture is the most reliable method although more fastidious to perform. The active compounds present in the green tea enriched extracts may act in synergy to inhibit the targeted cells and are worth investigating for antimicrobial applications in food preservation.

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