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Inhibitory Effects of Green Tea Polyphenols on the Production of a Virulence Factor of the Periodontal-Disease-Causing Anaerobic Bacterium *Porphyromonas gingivalis*

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The effect of polyphenolic compounds isolated from green tea (*Camellia sinensis*) on the production of toxic end metabolites of *Porphyromonas gingivalis* was investigated. Green tea polyphenols completely inhibited the production of *n*-butyric acid and propionic acid at a concentration of 1.0−2.0 mg/mL in general anaerobic medium (GAM). (−)-Epigallocatechin gallate (EGCg), which is a major component of tea polyphenols also inhibited the production of phenylacetic acid at 0.5 mg/mL in GAM broth. In the experiment using resting cells of *P. gingivalis*, phenylacetic acid was produced from L-phenylalanine and phenylpyruvic acid, but this reaction was also inhibited by EGCg, (−)-epicatechin gallate, and (−)-gallocatechin gallate. However, (+)-catechin, (+)-gallocatechin, (−)-epicatechin, and (−)-epigallocatechin did not inhibit those reactions. These results indicate that the inhibitory effect on the production of toxic end metabolites of *P. gingivalis* can be attributed to the presence of the galloyl moiety, which is ester-linked with the 3-OH of the catechin moiety in the polyphenolic compounds. This study shows that continuous application of tea polyphenols on a daily basis can be considered as a useful and practical method for the prevention of periodontal diseases.

KEYWORDS: Green tea polyphenols; (−)-epigallocatechin gallate; *Porphyromonas gingivalis*; periodontal disease; phenylacetic acid

INTRODUCTION

Periodontal diseases bring inflammatory and destructive lesions in periodontal tissues. These diseases are responsible for the bacteria in subgingival plaque. *Porphyromonas gingivalis* (formerly *Bacteroides gingivalis*) has been frequently isolated from the gingival pockets of patients with advanced adult periodontitis (*1*, *2*). *P. gingivalis* adheres to oral epithelial cells. Adherence of bacteria to their host tissue cells is the first step of the bacterial infection. The adhered bacteria begin their growth, colonization, and production of virulent factors that injure the host tissue cells (*3*). *P. gingivalis* has several periodontal pathogenic factors, including membrane-associated proteases, immunoactive cellular compounds, and cytotoxic metabolic end products (*4*−*6*).

Cytotoxic metabolic end products (butyric acid, propionic acid, phenylacetic acid, etc.) are produced by *P. gingivalis* in the periodontal pockets. These compounds easily penetrate into periodontal tissue, due to their low molecular weight, and subsequently disturb host cell activity and host defense systems at millimolar concentrations (*7*−*10*). These concentration levels were the same levels found in *P. gingivalis* culture supernatant and gingival crevicular fluid of periodontally diseased subjects (*11*). Furthermore, these compounds injure periodontal cells and result in degradation of tissues by bacterial proteinases. For these reasons, the inhibition of toxic end metabolite production is as important as the inhibition of growth and cellular adherence of *P. gingivalis* in preventing periodontal diseases.

Recent studies on the effects of green tea polyphenols (tea catechins) on dental caries showed that the polyphenols decreased caries risk in vitro (*12*, *13*) and in vivo (*14*, *15*). It was also reported that green tea polyphenols inhibit the collagenase activity (*16*), growth and cellular adherence of *P. gingivalis* (*17*), known as the virulence factors of periodontal disease.

Dental caries and periodontal disease are two major diseases occurring in the mouth. There are many reports about the influence of food constituents on dental caries causing bacteria (*7*−*10*, *18*, *19*), but very few studies reported with regard to periodontal disease. This paper reports the effects of green tea polyphenols on the production of toxic end metabolites of *P. gingivalis*, one of the virulence factors of periodontal diseases.

MATERIALS & METHODS

Bacterial Strains and Culture Conditions. *Porphyromonas gingivalis* 381, *Porphyromonas melaninogenicus* GAI 5596, and *Strep-*

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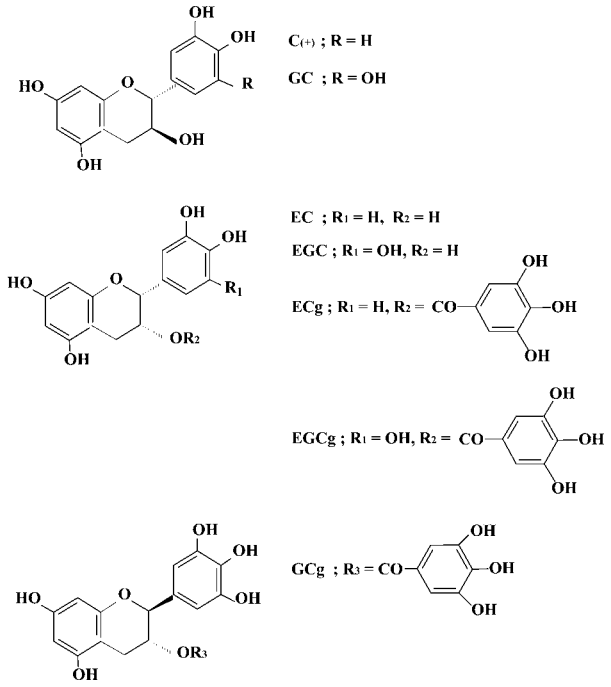

Figure 1. Structures of green tea polyphenols.

tococcus mutans MT8148 were used in this study. These bacteria were grown in general anaerobic medium (GAM, Nissui Medical Co., Tokyo, Japan) at 37 °C in an anaerobic atmosphere of 80% N_2, 15% CO_2, and 5% H_2.

Tea Polyphenol Preparations Examined and Chemicals Employed. The sample examined in this study was a mixture of green tea polyphenols, which was prepared by extracting Japanese green tea (*Camellia sinensis*) with hot water and then by partitioning with ethyl acetate, as reported previously (*12*). It was composed mainly of (+)-catechin ($C_{(+)}$, 3.5%), (−)-epicatechin (EC, 7.0%), (+)-gallocatechin (GC, 14.8%), (−)-epigallocatechin (EGC, 15.0%), (−)-epigallocatechin gallate (EGCg, 18.0%), (−)-epicatechin gallate (ECg, 4.6%), and (−)-gallocatechin gallate (GCg, 11.6%). For examination, each component of these polyphenolic compounds was also isolated in its pure state, by the method reported previously (**Figure 1**) (*12*). All other chemicals used were of reagent or analytical grade.

Analysis of Short-Chain Fatty Acids. Short-chain fatty acids of cell free culture filtrates were analyzed by a modification of the method of Miwa et al. (*20*), using high performance liquid chromatography (HPLC). Short-chain fatty acids in the cell free culture filtrate were converted to their respective 2-nitrohydrazides using 1-ethyl-3-(dimethyl amino propyl) carbodiimide hydrochloride for the HPLC analysis. A Waters 600E liquid chromatography with a Waters 490E multiwavelength detector (operating at 400 nm) and a Waters 741 data module were used. A YMC-Pack FA column (250- × 6-mm i.d., YMC, Kyoto, Japan) was maintained at 55 °C and eluted with acetonitrile/methanol/water (18:12:70 to 42:28:30) by a linear gradient system for 40 min at a flow rate of 1.2 mL/min.

Preparation of Resting Cells. Resting cells of *P. gingivalis* 381 were prepared by the method previously reported (*21*). *P. gingivalis* 381 was cultured for 48 h and harvested outside the anaerobic chamber by centrifugation at 8000*g* for 15 min. The cells were then washed three times in a 10 mM potassium phosphate buffer (pH 7.2) containing 10 mM $MgCl_2$ and 5 mM mercaptoethanol. The centrifugation and washing process resulted in nonsurvival of the pelleted bacteria as indicated by a lack of growth on GAM inoculated with the concentrated bacteria and incubated anaerobically for 5 days. The pellet of bacteria was stored at −80 °C.

Production of Fatty Acids by Resting Cells. For each experiment, a mixture of 0.2 mL of each substrate (37.5 mM), 1 mL of *P. gingivalis* resting cell suspension (18 mg/mL) and 0.3 mL of 10 mM potassium phosphate buffer (pH 7.2) was used. After 24 h incubation at 37 °C in a test tube, the mixture was centrifuged at 8000*g* for 10 min. The amount of fatty acid in the supernatant of the mixture was analyzed by HPLC as mentioned above.

RESULTS & DISCUSSION

Inhibition of Short-Chain Fatty Acid Production of *P. gingivalis*. *P. gingivalis* isolated from human periodontal pockets is known as a bacterium that produces cytotoxic metabolic end products (*22*). **Figure 2** shows that *P. gingivalis* 381 produces a variety of fatty acids in GAM broth, and their numbers and amounts are numerous in comparison with other oral bacteria, *S. mutans* MT8148 and *P. meraninogenicus* GAI 5596. The main cytotoxic end products, *n*-butyric acid and propionic acid were produced in the GAM broth. The production of these fatty acids was inhibited by green tea polyphenol mixture in dosage dependent manner (**Figures 3** and **4**). The production of *n*-butyric acid and propionic acid was inhibited at concentrations of tea polyphenol mixture of 0.5 mg/mL for 42 h and 1.0−2.0 mg/mL for 90 h.

Inhibitory Effect of EGCg on the Production of Phenylacetic Acid in GAM Broth. HPLC or gas chromatography of

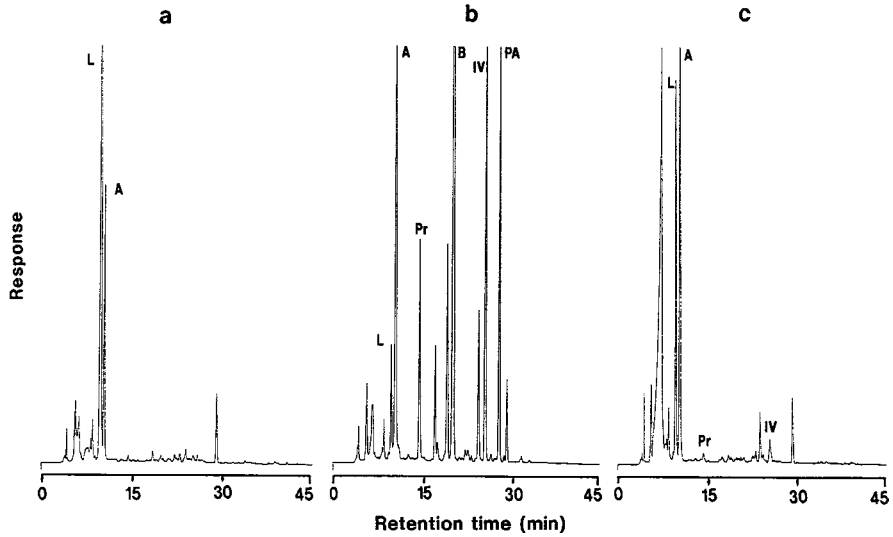

Figure 2. HPLC chromatogram of free metabolic acids from oral bacteria extracted from GAM broth after 72 h of anaerobic culture. L, lactic acid; A, acetic acid; Pr, propionic acid; B, *n*-butyric acid; IV, isovaleric acid; PA, phenylacetic acid. **a**, *S. mutans* MT8148; **b**, *P. gingivalis* 381; **c**, *P. melaninogenicus* GAI 5596.

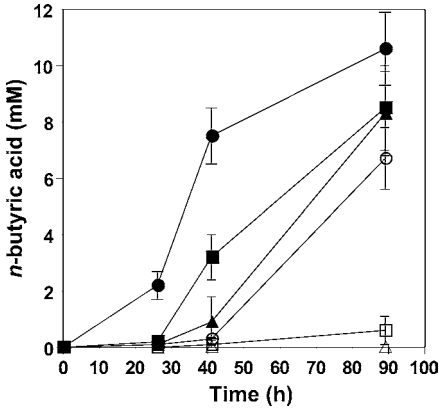

Figure 3. Inhibitory effect of tea polyphenols (TP) on the production of *n*-butyric acid in GAM broth. ●, 0 mg/mL of TP; ■, 0.125 mg/mL of TP; ▲, 0.25 mg/mL of TP; ○, 0.5 mg/mL of TP; □, 1.0 mg/mL of TP; △, 2.0 mg/mL of TP. Data are expressed as mean ± standard deviation ($n = 3$).

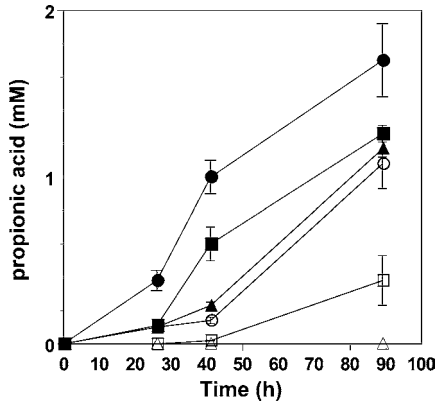

Figure 4. Inhibitory effect of tea polyphenols (TP) on the production of propionic acid in GAM broth. ●, 0 mg/mL of TP; ■, 0.125 mg/mL of TP; ▲, 0.25 mg/mL of TP; ○, 0.5 mg/mL of TP; □, 1.0 mg/mL of TP; △, 2.0 mg/mL of TP. Data are expressed as mean ± standard deviation ($n = 3$).

acidic end products in the medium is frequently used for the classification and identification of anaerobic bacteria. Phenylacetic acid is a characteristic end product of *P. gingivalis* in distinguishing it from other oral assacharolytic *Bacteroides* (*Porphyromonas*) species (*23*, *24*). As shown in **Figure 2**, *P. gingivalis* produces phenylacetic acid in GAM broth, but other oral bacteria that are not causing periodontal disease do not produce this acidic end product. This compound has been reported to exhibit cytotoxity in combination with other fatty acids (*n*-butyric acid or propionic acid) (*25*).

Growth of *P. gingivalis* was inhibited by EGCg, which is a major component of green tea polyphenols, and the CFU/ml of this bacterium in the broth was decreased in the concentration dependent manner. The production of phenylacetic acid in the broth was also inhibited by EGCg (**Figure 5**). However, it is noteworthy that the production of phenylacetic acid was almost completely inhibited at 0.5 mg/mL of EGCg, but at that time, survival cells of *P. gingivalis* were 2.7×10^6 CFU/ml in the same broth. This fact suggests that the amount of phenylacetic acid produced by *P. gingivalis* is not compatible with the survival cell number and that EGCg inhibits the pathway of toxic end products such as phenylacetic acid.

Phenylacetic Acid Production by Resting Cells of *P. gingivalis*. The production of phenylacetic acid was examined using resting cells of *P. gingivalis*. Resting cells lacked growth

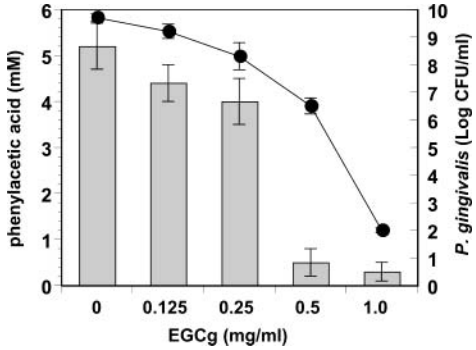

Figure 5. Inhibitory effect of EGCg on the production of phenylacetic acid and the growth of *P. gingivalis* 381 in GAM broth for 48 h anaerobically. Bar graph, phenylacetic acid; ●, *P. gingivalis* growth. Data are expressed as mean ± standard deviation ($n = 3$).

Table 1. Effect of Various Compounds on the Production of phenylacetic acid by resting cells of *Porphyromonas gingivalis*

compounds (5 mM)	penylacetic acid[a] (mM)
none	0.45 ± 0.12
L-phenylalanine	2.76 ± 0.34
D-phenylalanine	0.50 ± 0.16
3-phenylpropionic acid	0.32 ± 0.19
phenylpyruvic acid	3.10 ± 0.44
glycyl-L-phenylalanine	2.17 ± 0.29

ability but retained metabolic ability with respect to the production of phenylacetic acid from various substrates. **Table 1** shows resting cells of *P. gingivalis* produced phenylacetic acid from L-phenylalanine, phenylpyruvic acid, and glycyl-L-phenylalanine but not from D-phenylalanine or 3-phenylpropionic acid. Resting cell suspension controls, which are absent of added substrates, always produced a certain amount of phenylacetic acid, presumably owing to the action of proteases on cellular material and the subsequent metabolism of released L-phenylalanine. When resting cells were heated at 100 °C for 15 min, phenylacetic acid was absent even when L-phenylalanine or phenylpyruvic acid were added to the reaction mixture. It seems that no conversion of L-phenylalanine or phenylpyruvic acid into phenylacetic acid could have occurred under those conditions.

Inhibitory Effect of Tea Polyphenols on the Production of Phenylacetic Acid by Resting Cells of *P. gingivalis*. It is suggested that phenylpyruvic acid may be an intermediate in the conversion of phenylalanine to phenylacetic acid (*26*). Phenylacetic acid was actually produced from phenylpyruvic acid by resting cells of *P. gingivalis* in the experiment. This production was completely inhibited by EGCg, ECg, and GCg, but it was not inhibited by $C_{(+)}$, GC, EC, and EGC (**Table 2**). EGCg inhibited the production of phenylacetic acid not only from phenylpyruvic acid but also from L-phenylalanine and glycyl-L-phenylalanine (**Table 3**). Among the tea polyphenols, EGCg, ECg, and GCg had high inhibitory activities, while $C_{(+)}$, GC, EC, and EGC were not so effective. The polyphenol effective for inhibition of phenylacetic acid production was the catechin derivative which bears a galloyl moiety linked by an ester linkage (**Figure 1**).

In our previous papers, tea polyphenols containing a galloyl moiety were found to act as inhibitors to both glucosyltransferase of *Streptococcus mutans* and *Streptococcus sobrinus* (*13*), and collagenase of some eukaryotic and prokaryotic cells (*18*). Furthermore, the adherence of *P. gingivalis* on the host cells

Table 2. Effect of polyphenolic compounds on the production of phenylacetic acid from phenylpyruvic acid by resting cells of *Porphyromonas gingivalis*[a]

cpds	phenylacetic acid[b] (mM)
none	3.45 ± 0.38
$C_{(+)}$	3.51 ± 0.60
GC	3.48 ± 0.44
EC	3.50 ± 0.39
EGC	3.75 ± 0.51
ECg	0.11 ± 0.06
EGCg	0.06 ± 0.02
GCg	0.03 ± 0.01

[a] Concentration of compounds, 1.0 mg/ml; incubation time, 24 h. [b] Values are mean ± sstandard deviation ($n = 3$).

Table 3. Inhibitory effect of EGCg on the production of phenylacetic acid from various substrates by resting cells of *Porphyromonas gingivalis*[a]

substrate	concn of EGCg (mg/ml)	phenylacetic acid[b] (mM)
L-phenylalanine	0	3.04 ± 0.37
	0.5	2.00 ± 0.11
	1.0	1.56 ± 0.23
phenylpyruvic acid	0	3.67 ± 0.41
	0.5	0.73 ± 0.05
	1.0	0.08 ± 0.01
glycyl-L-phenylalanine	0	2.69 ± 0.29
	0.5	1.77 ± 0.15
	1.0	0.90 ± 0.04

[a] Incubation time, 24 h. [b] Values are mean ± standard deviation ($n = 3$).

was also inhibited by EGCg (*19*). It is thus evident that the inhibitory effects shown by tea polyphenols of gallate type are attributed to the structural contribution of the galloyl moiety linked by an ester linkage with 3-OH of catechin structural molecules.

This paper has shown that green tea polyphenols greatly reduce the ability of *P. gingivalis* to produce toxic end metabolites. This fact may indicate that green tea polyphenols or their components have the possibility to be applicable as a remedy for the prevention of periodontal diseases. It is noteworthy that a cup of green tea extract (100 mL) usually contains 50−150 mg of tea polyphenols (*27*, *28*). This concentration of tea polyphenols is nearly equal to those used in the experiments in this paper. A green tea extract is usually consumed during and after each meal as a custom in Japan, and a traditional saying states that green tea clean the mouth. This is supported by present and previous studies (*12*−*14*, *18*, *19*), since green tea polyphenols reduces the risk of dental caries and periodontal disease.

ABBREVIATIONS USED

$C_{(+)}$, (+) catechin; EC, (−) epicatechin; GC, (+) gallocatechin; EGC, (−) epigallocatechin; ECg, (−) epicatechin gallate; EGCg, (−) epigallocatechin gallate; GCg, (−) gallocatechin gallate; GAM, general anaerobic medium; HPLC, high-performance liquid chromatography.

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LITERATURE CITED

(1) Slots, J. The predominant cultivable microflora of advanced periodontitis. *Scand. J. Dent. Res*. **1977**, *85*, 114−121.
(2) Tanner, A.; Maiden, M. F.; Macuch, P. J.; Murray, L. L.; Kent, R. L., Jr. Microbiota of health, gingivitis, and initial periodontitis. *J. Clin. Periodontol*. **1998**, *25*, 85−98.
(3) Gibbons, R. J. Adherent interactions which may affect microbial ecology in the mouth. *J. Dent. Res*. **1984**, *63*, 378−385.
(4) Holt, S. C.; Kesavalu, L.; Walker, S.; Genco, C. A. Virulence factors of *Porphyromonas gingivalis*. *Periodontol. 2000*. **1999**, *20*, 168−238.
(5) Mayland, D.; Holt, S. C. Biology of asaccharolytic black-pigmented *Bacteroides* species. *Microbiol. Res*. **1988**, *52*, 134−152.
(6) Lamont, R. J.; Jenkinson, H. F. Life below the gum line: pathogenic mechanism of *Porphyromonas gingivalis*. *Microbiol. Mol. Biol. Rev*. **1998**, *62*, 1244−1263.
(7) Tometti, M.; Eftimiadi, C.; Damiani, G.; Buffa, P.; Buffa, D.; Botta, G. A. Short chain fatty acids present in periodontal pockets may play a role in human periodontal diseases. *J. Periodontal Res*. **1987**, *22*, 190−191.
(8) Singer, R. F.; Buchner, B. A. Butyrate and propionate: important components of toxic dental plaque extracts. *Infect. Immun*. **1981**, *32*, 458−463.
(9) Eftimiadi, C.; Stashenko, P.; Tonetti, M.; Mangiante, P. E.; Massara, R.; Zupo, S.; Ferrarini, M. Divergent effect of the anaerobic bacterial byproduct butyric acid on the immune response: suppression of T-lymphocyte proliferation and stimulation of interleukin-1 beta production. *Oral Microbiol. Immunol*. **1991**, *6*, 17−23.
(10) Kurita-Ochiai, T.; Fukushima, K.; Ochiai, K. Volatile fatty acids, metabolic byproducts of periodontopathic bacteria, inhibit lymphocyte proliferation and cytokine production. *J. Dent. Res*. **1995**, *74*, 1367−1373.
(11) Niederman, R.; Buyle-Bodin, Y.; Lu, B. Y.; Robinson, P.; Naleway, C. Short-chain carboxylic acid concentration in human gingival crevicular fluid. *J. Dent. Res*. **1997**, *76*, 575−579.
(12) Sakanaka, S.; Kim, M.; Taniguchi, M.; Yamamoto, T. Antibacterial substances in Japanese green tea extract against *Streptococcus mutans*, a cariogenic bacterium. *Agric. Biol. Chem*. **1989**, *53*, 2307−2311.
(13) Sakanaka, S.; Sato, T.; Kim, M.; Yamamoto, T. Inhibitory effects of green tea polyphenols on glucan synthesis and cellular adherence of cariogenic streptococci. *Agric. Biol. Chem*. **1990**, *54*, 2925−2929.
(14) Sakanaka, S.; Shimura, N.; Aizawa, M.; Kim, M.; Yamamoto, T. Preventive effect of green tea polyphenols against dental caries in conventional rats. *Biosci. Biotechnol. Biochem*. **1992**, *56*, 592−594.
(15) Otake, S.; Makimura, M.; Kuroki, T.; Nishihara, Y.; Hirasawa, M. Anticaries effects of polyphenolic compounds from Japanese green tea. *Caries Res*. **1991**, *25*, 438−443.
(16) Yanagida, A.; Kanda, T.; Tanabe, M.; Matsudaira, F.; Oliverira Cordeiro, J. G. Inhibitory effects of apple polyphenols and related compounds on cariogenic factors of mutans streptococci. *J. Agric. Food Chem*. **2000**, *48*, 5666−5671.
(17) Daglia, M.; Tarsi, R.; Papetti, A.; Grisoli, P.; Dacarro, C.; Pruzzo, C.; Gazzani, G. Antiadhesive effect of green and roasted coffee on *Streptococcus mutans'* adhesive properties on saliva-coated hydroxyapatite beads. *J. Agric. Food Chem*. **2002**, *50*, 1225−1229.
(18) Makimura, M.; Hirasawa, M.; Kobayashi, K.; Indo, J.; Sakanaka, S.; Taguchi, T.; Otake, S. Inhibitory effect of tea catechins on collagenase activity. *J. Periodontol*. **1993**, *64*, 630−636.
(19) Sakanaka, S.; Aizawa, M.; Kim, M.; Yamamoto, T. Inhibitory effects of green tea polyphenols on growth and cellular adherence of an oral bacterium, *Porphyromonas gingivalis*. *Biosci. Biotechnol. Biochem*. **1996**, *60*, 745−749.

(20) Miwa, H.; Hiyama, C.; Yamamoto, M. High-performance liquid chromatography of short- and long-chain fatty acids as 2-nitrophenylhydrazides. *J. Chromatogr*. **1985**, *321*, 165−174.
(21) Takahashi, N.; Sato, T.; Yamada, T. Metabolic pathways for cytotoxic end product formation from glutamate- and aspartate-containing peptides by *Porphyromonas gingivalis*. *J. Bacteriol*. **2000**, *182*, 4704−4710.
(22) Bourgeau, G.; Mayrand, D. Phenylacetic acid production by *Bacteroides gingivalis* from phenylalanine and phenylalanine-containing peptides. *Can. J. Microbiol*. **1983**, *29*, 1184−1189.
(23) Mayrand, D. Identification of clinical isolates of selected species of *Bacteroides*: production of phenylacetic acid. *Can. J. Microbiol*. **1979**, *25*, 927−928.
(24) Johansen, H.; Olsen, I.; Kerekes, K. Differentiation between *Bacteroides gingivalis*, *Bacteroides endodontalis* and *Bacteroides asaccharolyticus* by means of HPLC analysis of nonderivatized free metabolic acids. *Oral Microbiol. Immunol*. **1988**, *3*, 42−45.
(25) Grenier, D.; Mayrand, D. Cytotoxic effects of culture supernatants of oral bacteria and various organic acids on Vero cells. *Can. J. Microbiol*. **1985**, *31*, 302−304.
(26) Allison, M. J. Phenylalanine biosynthesis from phenylacetic acid by anaerobic bacteria from the rumen. *Biophys. Biochem. Res. Commun*. **1965**, *18*, 30−35.
(27) Maeda, S.; Nakagawa, M. General chemical and physical analyses on various kinds of green tea. *Chagyo Kenkyuhokoku* (in Japanese) **1977**, *45*, 85−92.
(28) Takeo, T.; Ikegaya, K.; Nakagawa, M. Cha no shinsahou. In *Shin-chagyo Zensyo* (in Japanese); Shizuoka-ken Chagyo Kaigisyo: Shizuoka, Japan, 1988; pp 393−420.

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