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Epigallocatechin-3-Gallate Decreases Plasma and Urinary Levels of *p*-Cresol by Modulating Gut Microbiota in Mice

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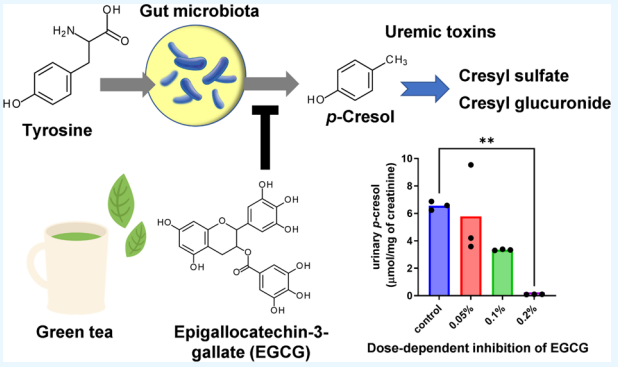
ABSTRACT: *p*-Cresol (PC), a gut bacterial product of tyrosine catabolism, is recognized as a uremic toxin that has negative biological effects. Lowering the plasma PC level by manipulating the gut bacterial composition represents a promising therapeutic strategy in chronic kidney disease. This study was conducted to reveal whether epigallocatechin-3-gallate (EGCG) decreases plasma PC levels by limiting its bacterial production in a mouse model. The PC concentration in the samples was measured by high-performance liquid chromatography (HPLC) after treatments with sulfatase and β-glucuronidase. The results showed that the addition of EGCG to the diet decreased the plasma and urinary concentrations of PC in a dose-dependent manner, with a statistically significant difference between the control group and the 0.2% EGCG group. However, once EGCG was enzymatically hydrolyzed to epigallocatechin (EGC) and gallic acid, such effects were lost almost completely. The addition of 0.2% EGCG in the diet was accompanied by a decreased abundance of Firmicutes at the phylum level and Clostridiales at the order level, which constitute a large part of PC produced from tyrosine. In conclusion, EGCG, not EGC, reduced plasma and urinary concentrations of PC in mice by suppressing its bacterial production with accompanying alteration of the relative abundance of PC producers.



INTRODUCTION

Uremic toxins are substances that interact negatively with biological functions. The accumulation of uremic toxins is associated with systemic disorders such as chronic kidney disease (CKD), endothelial dysfunction, insulin resistance, and cognitive impairment, leading to higher mortality and lower quality of life.[1] Phenol and *p*-cresol (PC) are typical uremic toxins generated from tyrosine by intestinal bacteria. They are taken into the blood circulation and then undergo conjugations with sulfation or glucuronidation in the liver. *p*-Cresyl sulfate (PCS) is considered to be a potential cause of excess cardiovascular disease and mortality in patients with CKD.[2] Mechanistically, it can trigger inflammation and oxidative stress in endothelial cells, contributing to endothelial dysfunction and arterial stiffness.[3] The excretion of PCS mainly depends on the versatile tubular transporter systems in the kidney, and limited renal clearance in patients with CKD leads to a progressive accumulation in the blood.[4] PCS is bound with a high affinity to plasma proteins and therefore is poorly removed by dialysis. Not only preserving renal excretory function but also inhibiting bacterial production of PC would also seem to be a useful way to lessen the disproportionate burden in patients with CKD. Some approaches to controlling PC production have been evaluated; for instance, the use of probiotics, prebiotics, and their combinations, is a topic of keen interest in lowering plasma levels of PCS.[5] Human intervention studies have provided evidence that certain types of dietary fiber bring about beneficial effects in CKD patients.[6−8] In general, treatments with prebiotics and probiotics aim to modulate gut microbiota based on the concept of increasing bacterial saccharolytic activity and limiting proteolytic activity in the large intestine.[9] Strategies for decreasing the bacterial production of PC show great promise as alternative treatments to mitigate the complications of CKD.

Recent studies also provide evidence that a certain type of polyphenols can modulate gut bacterial composition by bilaterally acting as an antimicrobial and stimulating growth.[10,11] Green tea is a dietary source of polyphenols, mainly epicatechin, epigallocatechin (EGC), epicatechin-3-gallate, and epigallocatechin-3-gallate (EGCG). EGCG is the most abundant polyphenol in green tea. Parts of orally ingested EGCG can reach the large intestine, which is inhabited by a wide variety of bacteria.[12,13] Green tea, or some of its

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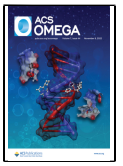

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Table 1. Food Intake, Body Weight, Feces Weight, and Cecal Digesta Weight[a]

	Exp. 1 (EGCG dose-dependent)				Exp. 2 (EGCG hydrolysate)		
	control	0.05%	0.1%	0.2%	control	EGCG	EGC + GA
number of mice	3	3	3	3	4	4	4
food intake (g/2 weeks)	101.2 ± 9.8	93.1 ± 8.6	100.4 ± 4.3	89.7 ± 1.3	91.1 ± 2.6	91.9 ± 9.6	90.3 ± 7.6
feces dry weight (g/2 weeks)	6.76 ± 0.56	6.09 ± 0.54	6.90 ± 1.14	6.68 ± 1.14	6.20 ± 0.26	6.12 ± 0.95	6.18 ± 0.59
initial body weight (g)	27.8 ± 1.7	27.7 ± 0.8	27.7 ± 0.5	27.4 ± 0.9	28.7 ± 0.8	28.9 ± 2.4	28.9 ± 1.2
final body weight (g)	43.0 ± 4.1	40.5 ± 3.6	41.6 ± 4.7	37.9 ± 0.3	42.7 ± 4.3	43.0 ± 6.3	43.5 ± 4.0
cecal digesta wet weight (g)	0.24 ± 0.05	0.29 ± 0.06	0.22 ± 0.08	0.41 ± 0.07*	0.21 ± 0.03	0.48 ± 0.04##	0.29 ± 0.10

[a]Data are expressed as mean ± standard deviation (SD). *$p < 0.05$ compared to the control group (Exp. 1). ##$p < 0.01$ compared to the control group and the EGC + GA group (Exp. 2).

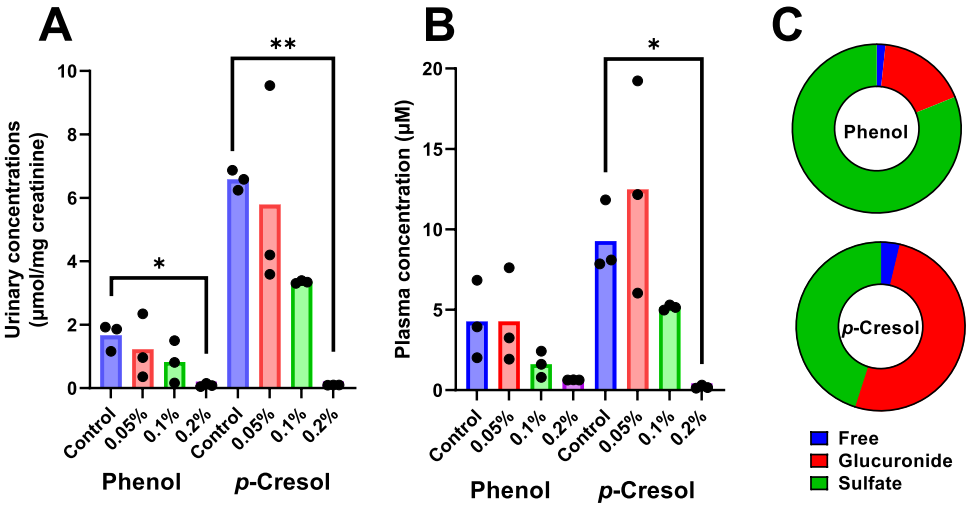

Figure 1. Dose-dependent decreases in urinary and plasma levels of uremic toxins derived from tyrosine. (Exp. 1). The urine of mice was collected during the last 2 days of the experiment. Conjugated forms were converted to the free forms by treating them with both sulfatase and β-glucuronidase. (A) Urinary concentrations of phenol and *p*-cresol as normalized to creatinine concentration. A bar represents its average value (n = 3). Each point in a group represents a value from a sample. (B) Plasma concentrations of phenol and *p*-cresol. (C) Profiles of conjugation with glucuronide and sulfate in mouse urine of the control group.

catechins, stimulates or hinders the growth of specific gut bacterial species,[14] and as a consequence of altered microbial composition, changes in the types and amount of microbially produced metabolites may occur. Therefore, we propose the hypothesis that EGCG influences the bacterial production of PC in the intestine by altering PC-producing bacteria. To test this hypothesis, the present work used animal experiments to evaluate the dose-dependent response (the first experiment, Exp. 1) and structure−activity (the second experiment, Exp. 2) of EGCG by measuring urinary and plasma concentrations of PC.

RESULTS

Food Intake and Body Weight. In both Exp. 1 and Exp. 2, there were no significant differences in food intake or dried feces weight in the 2-week feeding period (Table 1). There were also no significant differences in the final body weight, but the cecal digesta weight of the 0.2% EGCG-treated mice showed statistically significant increases.

Dose-Dependent Effect. The conjugated forms of phenol and PC were converted into free forms by enzymatic hydrolysis with sulfatase and β-glucuronidase, and the urinary and plasma levels of phenol and PC were measured as the total concentrations of their free and sulfate/glucuronide conjugation forms. In the control mice, the mean amount of PC excreted in the urine was 4 times higher than that of phenol (Figure 1A). The dietary addition of EGCG decreased urinary excretion of both compounds in a dose-dependent manner, with the levels in the urine of the mice fed the 0.2% EGCG diet being nearly undetectable. Statistical analysis demonstrated significant decreases in urinary phenol and PC in the 0.2% EGCG group compared to the control group ($p < 0.05$ for phenol and $p < 0.01$ for PC).

Total plasma concentrations of PC were also higher than those of phenol (Figure 1B). The mean plasma concentration of PC was 9.3 ± 2.2 μM for the control group and 0.2 ± 0.1 μM for the 0.2% EGCG group, a statistically significant difference ($p < 0.05$). Plasma phenol concentration exhibited a declining trend in line with the addition of EGCG, but it did not reach statistical significance.

Sulfate/Glucuronide Ratio of Conjugation. Phenol and PC in their free form were determined without an enzymatic hydrolysis reaction. The proportion of the free form to the total concentration of urinary phenol and PC constituted only a small percentage, with values of 1.7% for phenol and 3.7% for PC (Figure 1C). The amount of the glucuronide form was calculated by subtracting the free form from the data obtained after glucuronidase treatment. The amount of the sulfate form was also calculated by subtracting the free and glucuronide forms from the data obtained after glucuronidase plus sulfatase treatments. In the control mice, the sulfated form of phenol in the urine held a dominant share, accounting for 77% of the

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Table 2. Urinary Concentrations of Phenol and *p*-Cresol in Free and Conjugated Forms (Exp. 1)[a]

	control	0.05% EGCG	0.1% EGCG	0.2% EGCG
		Phenol (μmol/mg Creatinine)		
free	0.09 ± 0.02	0.12 ± 0.02	0.08 ± 0.03	0.06 ± 0.06
sulfate	1.28 ± 0.35	1.00 ± 0.91	0.65 ± 0.53	0.03 ± 0.02
glucuronide	0.28 ± 0.11	0.10 ± 0.10	0.10 ± 0.12	0.01 ± 0.02
total	1.65 ± 0.42	1.22 ± 1.02	0.82 ± 0.67	0.09 ± 0.05*
		p-Cresol (μmol/mg Creatinine)		
free	0.25 ± 0.06	0.29 ± 0.22	0.10 ± 0.02	0.01 ± 0.00
sulfate	2.95 ± 0.18	2.91 ± 2.06	1.59 ± 0.10	0.08 ± 0.01*
glucuronide	3.36 ± 0.36	2.57 ± 1.36	1.65 ± 0.15*	0.01 ± 0.00**
total	6.57 ± 0.31	5.78 ± 3.28	3.34 ± 0.04	0.10 ± 0.00**

[a]Data are expressed as mean ± SD. *$p < 0.05$ and **$p < 0.01$ compared with the control group.

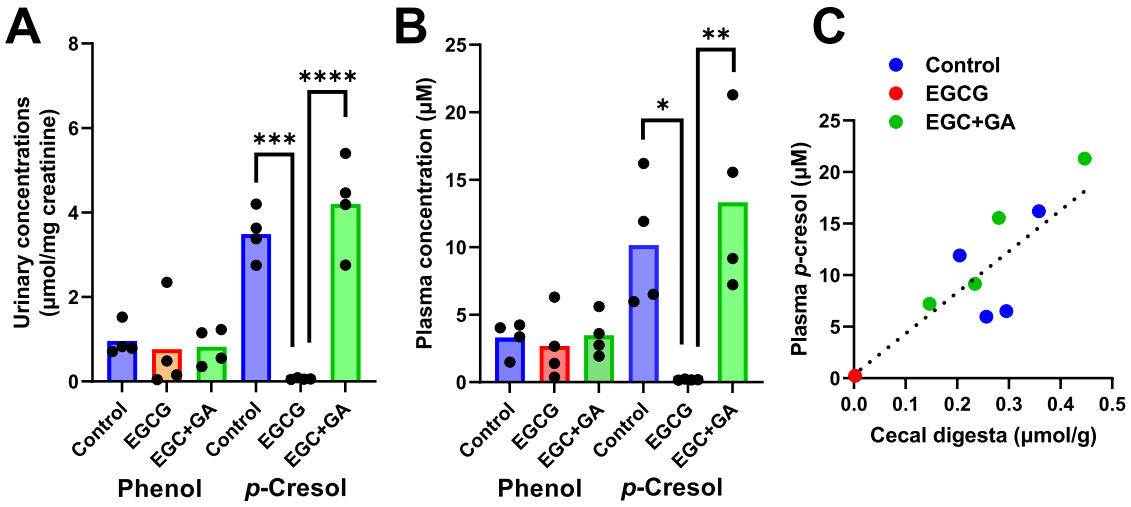

Figure 2. *p*-Cresol-lowering effect of EGCG was lost by hydrolysis to EGC and GA. (Exp. 2). The urine of mice was collected during the last 2 days of the experiment. (A) Urinary concentrations of phenol and *p*-cresol as normalized to creatinine concentration. A bar represents its average value ($n = 4$). Each point in a group represents a value from a sample. (B) Plasma concentrations of phenol and *p*-cresol. (C) Positive correlation of plasma concentration of *p*-cresol with its level in cecal digesta. Each mouse from the group was plotted on a graph.

Table 3. Cecal Concentrations of Phenol and *p*-Cresol[a]

	Exp. 1 (EGCG dose-dependent)				Exp. 2 (EGCG hydrolysate)		
	control	0.05%	0.1%	0.2%	control	EGCG	EGC + GA
				Cecal Digesta			
phenol (μmol/g)	0.15 ± 0.04	0.07 ± 0.02*	0.06 ± 0.05*	0.02 ± 0.00**	0.12 ± 0.09	0.04 ± 0.04	0.11 ± 0.07
p-cresol (μmol/g)	0.13 ± 0.02	0.16 ± 0.05	0.15 ± 0.09	0.01 ± 0.00*	0.28 ± 0.07	0.00 ± 0.00##	0.28 ± 0.13

[a]Data are expressed as mean ± SD. *$p < 0.05$ and **$p < 0.01$ compared to the control group (Exp. 1). ##$p < 0.01$ compared to the control group and the EGC + GA group (Exp. 2).

total concentration. Yet, at the same time, the sulfated form of PC accounted for 45%, showing a different trend a bit lower than the glucuronide form (51%). In mice fed the diets with EGCG at 0.05 and 0.1%, regardless of the additive amount of EGCG, the proportions of the sulfated or glucuronide forms to the total urinary concentration were almost consistent with the mice fed the control diet (Table 2). The proportions of the conjugated forms in the urine of mice fed the 0.2% diet did not follow such a pattern.

Ineffectiveness of EGCG Hydrolysate. In Exp. 2, the dietary addition of EGCG at 0.2% decreased the total urinary concentration of PC ($p < 0.001$), but this effect was completely lost with the hydrolysis of EGCG (Figure 2A). Between EGCG and its hydrolysate (an equimolar mixture of EGC and gallic acid (GA)), there was a statistically significant difference in the total urinary concentration of PC ($p < 0.0001$). The case was somewhat different for phenol; urinary phenol concentration did not show significant differences among the groups.

Such outcomes appeared consistently in the plasma concentrations. Total PC concentration in the plasma of mice fed the control diet, the EGCG diet, and the EGC + GA diet was 10.8 ± 5.2, 0.2 ± 0.1, and 12.4 ± 8.0 μM, respectively (Figure 2B). The EGCG group had significantly lower levels than other groups ($p < 0.05$ against the control group and $p < 0.01$ against the EGC + GA group). There were no significant differences in plasma phenol concentration among the groups.

Cecal Levels of Phenol and PC. The concentrations of phenol and PC in their free form per wet weight of cecal digesta are shown in Table 3. In both experiments, the cecal digesta of mice fed the 0.2% EGCG diet contained almost no PC, a statistically significant difference with the control group ($p < 0.05$ for Exp. 1 and $p < 0.01$ for Exp. 2). EGCG

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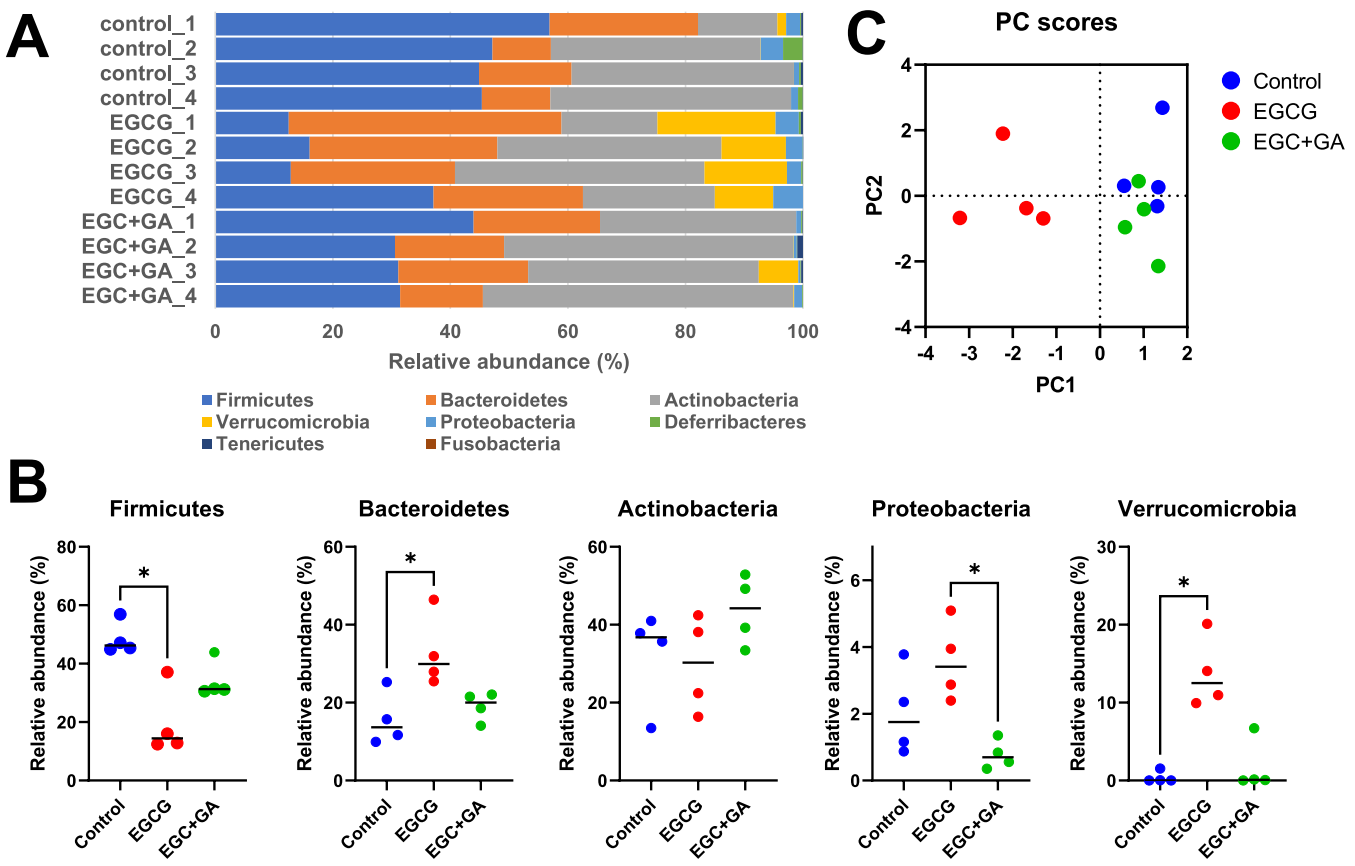

Figure 3. Microbial composition at the phylum level (Exp. 2). Feces were collected during the 2nd week of feeding, and the fecal bacterial taxonomic composition was determined by 16S metagenome sequencing. (A) Bar charts of the taxa abundance of each mouse. (B) Relative abundance of major phyla. A horizontal line represents the median value. Each point in a group represents a value from a sample. *$p < 0.05$ by Dunn's test for multiple comparisons. (C) Principal component analysis. Each mouse from the group was plotted on the first two principal components of the OTU profiles.

hydrolysate had little impact on the cecal level of PC. Figure 2C shows that the relationship between the cecal concentration of PC was closely correlated with its plasma concentration. The concentrations of PC both in the plasma and the cecal digesta in the EGCG group were at almost zero levels. In the case of phenol, a dose-dependent decrease was seen in Exp. 1, but dietary addition of EGCG did not lead to a statistically significant difference in Exp. 2.

Modulation of Microbial Composition. In Exp. 2, the four main phyla (Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria) represented 98.2% of the sequences in the feces of control mice (Figure 3A). In every mouse in the control group, the phylum Firmicutes was the most abundant. The addition of 0.2% EGCG in the diet provided a significant reduction in the relative abundance of Firmicutes compared to the control group ($p < 0.05$), whereas the EGC + GA diet had no effect (Figure 3B). The phyla Bacteroidetes and Verrucomicrobia were inversely increased ($p < 0.05$ for both), but in the case of EGCG hydrolysate, there was no practical impact. Principal component analysis (PCA) partially explained much of the variation (PC1, 38.4%, and PC2, 23.4%); the phylum taxonomic profiles of mice fed the control diet and the EGC + GA diet located on the near side, whereas the EGCG diet produced to a different profile (Figure 3C).

At the order level, bacteria of 12 orders were detected in the feces of the control group, of which 96.3% were represented by bacteria belonging to Clostridiales, Lactobacillales, Erysipelotrichales, Bacteroidales, Bifidobacteriales, Eggerthellales, and Enterobacterales (Figure 4A). Comparisons among the experimental groups for the top five orders and Verrucomicrobiales in the total sequence are shown in Figure 4B. EGCG treatment caused a downward shift in the relative abundance of the order Clostridiales, a member of phylum Firmicutes, but order Bacteroidales, a member of phylum Bacteroidetes, shifted upward, with significant differences from the control group ($p < 0.01$ for Clostridiales, $p < 0.05$ for Bacteroidales). Orders Lactobacillales and Erysipelotrichales, which also belong to the phylum Firmicutes, did not show significant differences. Interestingly, the relative abundance of Verrucomicrobiales rose precipitously in the EGCG group.

DISCUSSION

In recent studies, polyphenol-rich dietary sources have received much attention for their impact on elevating or depressing the bacterial production of metabolites while also modifying the gut microbial composition. Once orally consumed, parts of polyphenols reach the large intestine, where they have direct contact with a vast variety of bacteria. As a consequence of the modification of the bacterial community by polyphenols, metabolite production could be up- or downregulated. Short-chain fatty acids are an example of bacterial metabolites. Green tea polyphenols suppress their production in the intestine, but black tea polyphenols conversely increase them.[13] Our previous paper also reported that the diet addition of EGCG

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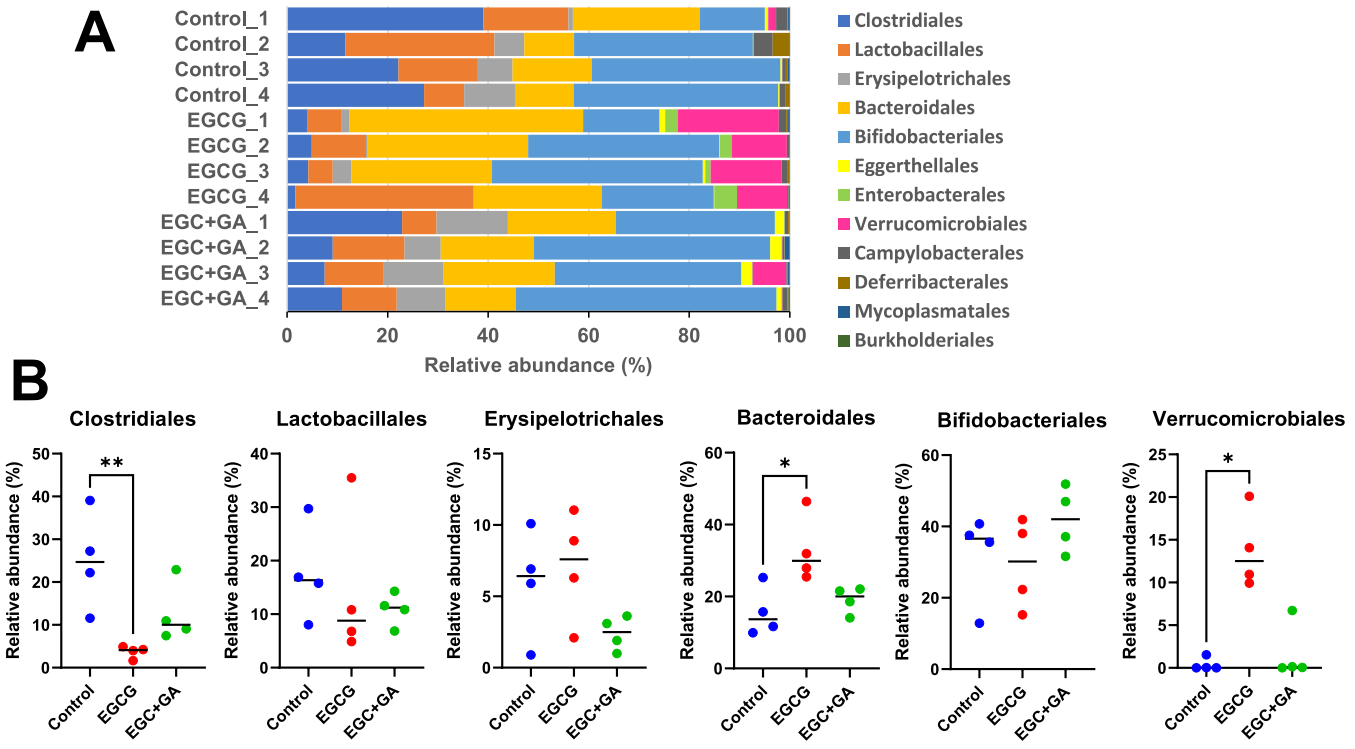

Figure 4. Microbial composition at the order level (Exp. 2). (A) Bar chart of the taxa abundance of each mouse. (B) Relative abundance of a major order. A horizontal line represents the median value. Each point in a group represents a value from a sample. *$p < 0.05$ and **$p < 0.01$ by Dunn's test for multiple comparisons.

decreased the cecal PC level in rats,[12] but whether EGCG has a significant impact on the plasma and urinary levels of phenol and PC via direct modulation of their producers in the gut remains unsettled. In addition, the impacts of tea polyphenols in a gallate form or a nongallate form on the relative changes of PC producers have not been systematically compared. Here, we evaluated the effects of EGCG and its hydrolysate (the mixture of EGC and GA) on urinary and plasma PC levels in healthy mice in relation to their modulation effect against PC producers.

It is well recognized that phenol and PC in urine and plasma consist largely of conjugated forms. In this study, the conjugated forms of phenol and PC were hydrolyzed by sulfatase and β-glucuronidase to convert them back into their free form, and then, the compounds were purified by the solid-phase extraction (SPE) method. In Exp. 1, the concentrations of PC in urine and plasma were decreased in response to the amount of EGCG added to the diet. This result provides direct evidence that EGCG has a beneficial effect on the suppression of the bacterial production of PC. Especially given that the cecum is one of the major organs where gut bacteria produce this uremic toxin, the finding that the cecal concentration of PC significantly decreased in the 0.2% EGCG group offers conclusive evidence to support the theory. It is reasonable to say that EGCG decreases the plasma and urinary concentrations of PC by reducing their production in the intestine. Next, to evaluate the respective ratio of conjugation with glucuronide and sulfate, we calculated the urinary concentrations of *p*-cresyl glucuronide (PCG) by subtracting the free form from the data obtained after hydrolysis only with β-glucuronidase. The concentration of PCS was also calculated by subtracting the concentrations of the free form and PCG from the total concentrations. It has been reported that PCG and PCS are found almost equally in rodents.[15] The same was true in this Exp. 1 of this study, which showed that the urine collected from the control mice had almost an equal percentage of glucuronate and sulfated forms. Since the urine of mice fed the 0.1% EGCG diet also maintained a balanced proportion similar to that in the control mice, it is reasonable to suggest that 0.1% EGCG exerted little influence on the conjugating reaction of PC in the liver.

Green tea polyphenols are divided into two main classes: one is catechins having a galloyl moiety and the other is catechins not having a galloyl moiety.[16] To reveal the structure−activity relationship between the galloyl type and the nongalloyl type, we next conducted another animal study (Exp. 2). Mice consumed either a diet containing EGCG at the 0.2% concentration or a diet containing the equimolar preparation of EGC and GA (prepared by the enzymatic hydrolysis of EGCG). The results demonstrated that the mice fed the 0.2% EGCG diet had markedly lower concentrations of PC in urine and plasma compared to the control diet, but the mice fed the EGC + GA diet excreted a significant amount of PC in the urine. This observation clearly shows that EGC was ineffective in reducing the bacterial production of PC, implying that the attachment of galloyl moiety to the structure of flavan-3-ol plays an important role. On the one hand, it is known that a part of EGCG entering the large intestine is hydrolyzed to EGC and GA by intestinal bacteria.[17] With the hydrolysis of EGCG, it may become progressively less effective, but the rest of EGCG can play a role in exerting the intended effect.

Tyrosine is microbially metabolized to 4-hydroxyphenylacetate and then converted into PC by 4-hydroxyphenylacetate decarboxylase (4-Hpd).[18] In a recent study by Saito et al.,[19] *Blautia hydrogenotrophica*, *Clostridium difficile*, *Romboutsia lituseburensis*, which are members of the order Clostridiales

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(heterotypic synonym of Eubacteriales, according to the NCBI Taxonomy Database), and *Olsenella uli*, a member of the order Coriobacteriales, were identified as major PC producers. These four PC-producing bacteria harbor a homolog of 4-Hpd. Amaretti et al.[20] also found that the families Lachnospiraceae and Ruminococcaceae, which are members of the order Clostridiales, had relevance to the production of PC. In light of this knowledge, it seems reasonable to predict that bacteria belonging to the order Clostridiales have a major role in producing PC in the gut. To find out whether the ingestion of EGCG induces an effect on PC-producing bacteria, we also determined the bacterial compositional change based on the taxonomic category. The results showed that the addition of 0.2% EGCG in the diet brought about a significant decrease in the relative abundance of the phylum Firmicutes. Of the major members constituting the phylum Firmicutes, only the order Clostridiales showed a statistically significant decrease as a result of EGCG. The relative abundance of the orders Lactobacillales and Erysipelotrichales could not be influenced by EGCG. A potential explanation for this is that some of the ingested EGCG reached the large intestine and reduced the abundance of the order Clostridiales exclusively, consequently suppressing PC production. However, whether EGCG interferes with enzyme reactions via direct inhibition of bacterial 4-Hpd is not known yet.

A vast variety of bacteria with phenol-producing ability seem to reside in the intestine commonly. Saito et al.[19] also identified some types of bacteria belonging to the orders Clostridiales, Fusobacteriales, and Enterobacterales that are capable of effectively producing phenol from tyrosine. These are phylogenetically classified in the phylum Firmicutes, Fusobacteria, and Proteobacteria, respectively. As explained above, EGCG was able to reduce the relative abundance of the phylum Firmicutes, but it showed a reverse trend for Proteobacteria. EGCG might function more to increase the abundance of Proteobacteria than to decrease it. Such a diversified range of phenol producers led us to suppose that EGCG could not by itself meaningfully reduce urinary and plasma levels of phenol. There is a need for more detailed studies to investigate the role of EGCG against phenol-producing bacteria.

Dietary supplementations with polyphenol-rich plant extracts may offer an opportunity to control the production of certain uremic toxins. For example, the consumption of a mixture of red wine and grape juice extracts for 4 days brought about a clinical advantage in reducing colonic protein fermentation or changing microbial amino acid metabolism, particularly a reduction of urinary PC.[21] In another study, supplementation with cranberry dry extract (daily dose of 1000 mg) for 2 months did not reduce the plasma levels of PCS in non-dialysis CKD patients.[22] The present study demonstrated a possible beneficial effect of green tea polyphenols on reducing bacterial production of PC in a mouse model. The dietary addition of EGCG had a strong reducing effect on urinary and plasma PC levels with a decreased abundance of PC producers in fecal microbiota. Based on the amount of food intake throughout the experimental period, the daily consumption of EGCG in the 0.2% EGCG group was calculated to be 305 mg/kg body weight of mice. This could be converted to the human equivalent dose at 24.7 mg/kg.[23] If efficient ways were devised to help a greater amount of EGCG reach the large intestine, it should be possible to reduce the dosage of EGCG to some extent.[24] Given that the microbial production of PC has been linked to a significant risk of cardiovascular mortality in CKD patients, EGCG may be a candidate agent for the treatment of the disease. In fact, EGCG has been studied for potential use in the management and prevention of various kidney diseases, with the major mechanisms of action associated with the reduction of oxidative stress and inflammation.[25] The present study looked at the beneficial health effect from a different perspective, focusing on uremic toxin control. Further investigation is warranted to elucidate the clinical benefit to which an EGCG-microbiota interaction is attributed.

CONCLUSIONS

This study demonstrated that dietary addition of EGCG reduced the plasma level and urinary excretion of PC in mice. The addition of 0.2% EGCG in the diet was accompanied by decreased abundance of PC-producing bacteria in the feces. However, once EGCG was hydrolyzed to EGC and GA, such effects were lost almost completely. Thus, the intervention of EGCG, not EGC, is a promising strategy for the prevention of disorders derived from this uremic toxin.

METHODS

Chemicals. Standards of phenol, PC, and *p*-chlorophenol were purchased from Fujifilm Wako Pure Chemical Co. (Osaka, Japan). Both β-glucuronidase from *Helix pomatia* and sulfatase from abalone entrails were purchased from Sigma-Aldrich Japan K.K. (Tokyo, Japan). A commercial EGCG product (>94% of purity) was obtained from DSM Nutrition Japan, K.K. (Tokyo, Japan). EGCG hydrolysate (a mixture of EGC and GA) was prepared by enzymatic hydrolysis of EGCG according to a previous procedure with a slight modification.[26] One gram of enzyme preparation (tannase-KTFHR, Kikkoman Co., Chiba, Japan), which consisted of 0.9% (w/w) tannase from *Aspergillus oryzae*, 99.0% glucose, and 0.1% inositol, was added to an aqueous solution of EGCG (10 g/L) and incubated at 37 °C for 60 min. The reaction mixture was evaporated and freeze-dried. The disappearance of EGCG after tannase treatment was confirmed by high-performance liquid chromatography (HPLC) (Figure S1). Except for EGC and GA, newly generated peaks were undetectable.

Animals and Diets. In Exp. 1, a total of 12 male ICR mice (4 weeks old) were purchased from Tokyo Laboratory Animals Science Co., Ltd. (Tokyo, Japan) and acclimated for 3 days in stainless steel metabolic cages at 22 °C in a room with an automatically controlled 12 h lighting cycle. During the acclimation period, the mice were fed the AIN93G formulation diet. They were then divided into four groups (n = 3 per group) according to their body weight and fed respective diets: a control diet, a 0.05% (w/w) EGCG diet, a 0.1% EGCG diet, or a 0.2% EGCG diet (Table S1). They were given free access to their experimental diets and tap water for 2 weeks. Feces were collected throughout the experimental period, and urine was collected during the last 2 days of the experiment. The feces were freeze-dried and stored at −40 °C. The mice were humanely killed by inhalation of high levels of carbon dioxide, and blood was immediately collected from the abdominal vein. Plasma was obtained after centrifuging at 2000g for 10 min and stored at −40 °C in a plastic microtube. The cecum was excised, and the cecal digesta was also collected and stored at −40 °C until use.

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In Exp. 2, we again purchased a total of 12 male ICR mice from the same breeder. After an acclimation period of 3 days, the mice were divided into three groups (n = 4 per group) and fed respective diets, a control diet, an EGCG diet, or an EGC + GA diet (Table S1) for 2 weeks. Feces and urine were collected as in Exp. 1. Blood and cecal digesta were collected on the final day of the experiment. All experiments were approved by the Committee for the Use and Care of Experimental Animals of Tokyo Kasei Gakuin University (approval number 2-11).

Analyses of Phenol and PC in Urine, Plasma, and Cecal Digesta. *Urine.* Thawed urine was diluted 50-fold with distilled water. For the measurement of the total amounts of phenol and PC (the sum of free, sulfate, and glucuronide forms), 50 μL of the diluted urine sample was first reacted with 20 units of sulfatase solution in 0.1 mL of 0.1 M acetate buffer (pH 5.0) at 37 °C for 2 h and second with 100 units of β-glucuronidase solution in 0.9 mL of 0.1 M phosphate buffer (pH 6.8) containing 5 mM sodium chloride at 37 °C for 15 min. For the glucuronide forms, the diluted urine sample (50 μL) was reacted only with 100 units of β-glucuronidase solution in 0.9 mL of the same phosphate buffer at 37 °C for 15 min. For the free form, the diluted urine sample proceeded without enzyme treatments.

After adding 10 μL of 0.5 mM *p*-chlorophenol solution as an internal standard, the reaction mixture was directly applied to a polymer-based SPE cartridge (Strata-X, particle size of 33 μm, Phenomenex, Inc., CA), which had been preconditioned with 1 mL of water, 1 mL of methanol, and 1 mL of water again. The cartridge was washed with 1 mL of water and 1 mL of 20% (v/v) aqueous methanol and then successively eluted phenol and PC with 1 mL of methanol. After passing through a 0.45 μm filtration membrane, 10 μL of the resulting filtrate was injected into an HPLC system (Shimadzu Co., Kyoto, Japan) that consisted of dual pumps (model LC-20 AD), an autosampler (model SIL-10A), a column oven (model CTO-20A), a fluorescence detector (model RF-20A), and a system controller (model SCL-10A). An analytical column (Unison UK-3C$_{18}$, 100 mm × 4.6 mm i.d., Imtakt, Co., Kyoto, Japan) was used for separation. The fluorescence detector was set at wavelengths of 270 nm for emission and 305 nm for excitation.[27] Gradient elution was performed by varying the proportion of solvent A (methanol−water, 25:75 v/v) to solvent B (methanol), at a flow rate of 1 mL/min. The mobile phase composition started at 100% solvent A (0% solvent B), after which the ratio of solvent B was linearly increased to 50% over 15 min, followed by a further increase of solvent B to 70% over 2 min. The composition was then brought back to the initial conditions over 2 min for the next run.

The measured values were normalized to the urinary creatinine concentration. Urinary creatinine concentrations were measured by the Jaffé assay.[28]

Plasma. Fifty microliters of thawed plasma was sequentially treated with 20 units of sulfatase in 0.1 mL of 0.1 M acetate buffer (pH 5.0) at 37 °C for 2 h and then with 100 units of β-glucuronidase in 0.1 M phosphate buffer (pH 6.8) containing 5 mM sodium chloride at 37 °C for 15 min. The subsequent procedure for SPE and HPLC measurements was the same as for urine.

Cecal Digesta. To prepare the homogenate, an aliquot of thawed cecal digesta was added to four volumes of distilled water. The homogenate (0.1 mL) was mixed with 0.4 mL of methanol and then centrifuged at 2000*g* and 4 °C for 10 min. The supernatant (0.4 mL) was diluted with 3.6 mL of water, and the SPE and HPLC analysis proceeded according to the method described above.

16S rRNA Gene Amplicon Sequencing. DNA was extracted from feces and PCR was performed according to the paper by Takahashi et al.[29] The V3−V4 regions of the 16S rRNA gene were amplified by PCR with universal primers 341F (5′-CCTACGGGAGGCAGCAG-3′) and 805R (5′-GGACTACCAGGGTATCTAAT-3′). Sequencing was conducted using a paired-end modified to a 600 bp cycle run on a MiSeq sequencing system with a MiSeq Reagent Kit version 3 (Illumina, Inc., San Diego, CA). The paired-end reads for each sample were joined using Fastq-join[30] and then processed with quality filtering with the FASTX-Toolkit.[31] The quality of the sequences was checked, and the passed sequences were clustered into operational taxonomic units (OTUs) with 97% pairwise identity. Taxonomic annotation of the representative sequence was performed using the Microbial Identification Database NGS-DB-BA 16.0 (TechnoSuruga Laboratory, Shizuoka, Japan).[32]

Statistical Analysis. p values less than 0.05 were considered significant. In Exp. 1, the statistical analysis was performed by Dunnett's test to compare the difference with the control group. If the data were not normalized, Dunn's test was adopted. In Exp. 2, statistics were calculated using one-way ANOVA, followed by the Tukey test. For the comparison of microbiota abundance, a nonparametric approach with Dunn's test involving pairwise comparisons was employed. All statistical analyses were conducted using GraphPad Prism version 9.03 (GraphPad Software, San Diego, CA).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c04731.

HPLC chromatogram of EGCG and its hydrolysate (Figure S1) and composition of diets (Table S1) (PDF)

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Author Contributions

T.U. designed the study, executed all animal experiments, and wrote the manuscript. M.I. was involved in the preparation of EGCG hydrolysate and helped to edit the manuscript. The authors reviewed and approved the final manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

CKD, chronic kidney disease; PC, *p*-cresol; PCS, *p*-cresyl sulfate; EGC, epigallocatechin; EGCG, epigallocatechin-3-gallate; GA, gallic acid; PCA, principal component analysis; SPE, solid-phase extraction; PCG, *p*-cresyl glucuronide; 4-Hpd, 4-hydroxyphenylacetate decarboxylase; HPLC, high-performance liquid chromatography

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