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# Agarwood (*Aquilaria Crassna*) Extracts Decrease High-protein High-fat Diet-induced Intestinal Putrefaction Toxins in Mice

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## Abstract

Agarwood (*Aquilaria spp.*) is famous for its aromatic resin, but its leaves are also prized as a healthy tea in South East Asia. Previously, we reported that agarwood extract (*Aquilaria sinensis* and *Aquilaria crassna*) shows laxative effect *via* acetylcholine receptors in constipation model mice and rats. In the present study, we investigated the effects of agarwood (*Aquilaria crassna*) on intestinal toxins, such as indole derivatives and ammonium to investigate the enteral environment. Male mice received regimens of three types of food, CE-7 (normal diet), CE-2 (high-protein normal diet), and Quick Fat (high-protein high-fat diet). Extracts of agarwood (water extract of agarwood: WEA and ethanol extract of agarwood: EEA) were orally administered once daily for a week. We measured the contents of indole derivatives and ammonium in feces and also examined the Minimum Inhibitory Concentrations (MICs) of agarwood against nine strains of enterobacteria *in vitro*. As compared with CE-7, Quick Fat increased the contents of indoles and ammonium in fecal beads. Single administration and multiple administrations for 7 days of WEA at 1,000 mg/kg/day decreased the contents of indoles and ammonium in fecal beads; on the other hand, multiple administrations of EEA decreased contents of indoles, but not those of ammonium. Interruption of administration abolished the effects of WEA and EEA. Quick Fat delayed carmine egestion in the digestive tract and administration of WEA and EEA accelerated the carmine egestion. Both WEA and EEA showed significant antimicrobial activity against some urease-positive bacteria, such as *Staphylococcus aureus*, *Clostridium difficile*, and *Bacteroides spp*. In conclusion, feeding with Quick Fat (high-protein high-fat diet) increased fecal-containing toxins and delayed carmine egestion in mice. Administrations of WEA and EEA decreased fecal-containing toxins and accelerated carmine egestion, and the decrement of fecal-containing toxins was abolished in response to interruption of the administration.

**Keywords:** Intestinal toxin; Intestinal putrifaction; Indole; Ammonium; Agarwood

**Abbreviations:** WEA: Water Extract of Agarwood; EEA: Ethanol Extract of Agarwood

## Introduction

Agarwood (*Aquilaria spp.*) is well known for its aromatic resin. More than fifteen botanical species called "Agarwood" exist: *Aquilaria apiculina*, found in the Philippines, *Aquilaria baillonil* in Cambodia, *Aquilaria baccarain* in Indonesia, *Aquilaria brachyantha* in Malaysia, *Aquilaria crassna* in Thailand, Malaysia, and Cambodia, and *Aquilaria sinensis* in Taiwan. Their leaves are also cultivated as a healthy tea in South East Asian countries such as Viet Nam, Laos, and Thailand. We previously reported that ethanol extract of *Aquilaria sinensis* or *Aquilaria crassna* induces laxative effect on loperamide-induced constipation model mice and low-fiber-diet-induced constipation model rats; extract of agarwood increases the contractional tension of isolated jejunum and ileum of guinea pigs; and extract of agarwood accelerates carmine egestion delayed by low fiber-diet [1-3]. Subsequently, we identified mangiferin and genkuwanin-5-*o*-*beta*-glucoside as the major constituents that give rise to the laxative effects of *Aquilaria sinensis* and *Aquilaria crassna*.

Plant origin phenolic compounds (phenolics from tea, olives, and wine) have been reported to have antimicrobial activities against *Escherichia coli*, *Salmonella*, and *Escherichia strains* [4-9]. Agarwood is rich in various types of polyphenols [10-12]. Agarwood (*Aquilaria sinensis*) has also been reported to show antimicrobial activities against *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis* [13]. Antimicrobial activities and laxative effect are closely associated with intestinal putrefaction, i.e. production of intestinal toxins, such as ammonium, indole, skatole, and p-cresol.

Ammonium is synthesized by urease-producing bacteria (*Bacteroides fragilis*, *Proteus vulgaris*, *Eubacterium aerofaciens*, *Clostridium difficile*, *Clostridium perfringens*, *Peptostreptococcus prevotii*, and *Lactobacillus fermentum*) in the large intestine. Ammonium synthesized in the intestines is absorbed into the portal vein and reaches the liver to be metabolized immediately, but hepatocirrhosis, hepatic function disorder, causes hyperammonemia. Hyperammonemia causes neurotoxicity and disruption of neurotransmission and is conventionally recognized as one of the sources of hepatic encephalopathy and Alzheimer's disease *via* attrition of reactive astrocyte and decrease of astroglia-derived glutathione [14-20]. Synthesized ammonium directly

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damages various organs including the gastrointestinal tract and induces arterioscleorosis, hypertensive, and autoimmune diseases.

Indolic compounds [(indole, skatole (3-methl indole), and IAA (indole 3-acetic acid)] are synthesized from tryptophan by indole-positive bacteria (*Escherichia coli*, *Escherichia coli* O-157, *Proteus vulgaris*, *Clostridium perfringens*, and *Morganella morganii*) mainly in the large intestine. Indolic compounds are absorbed into the portal vein and metabolized to the 3-indoxylsulfuric acid in the liver. 3-Indoxysulfuric acid is recognized as the most toxic compound to uremia patients and is also reported to inhibit osteoclast differentiation, bone-resorbing activity, smooth muscle proliferation, and HIFs-(hypoxia-inducible transcription factors) induced erythropoietin production [21-23]. Indole, normal indole, is toxic for lactic acid bacterium strains [24].

Productions of intestinal toxins such as indole, ammonium, skatole (3-metyl indole), or *p*-cresol, are directly influenced by nutritional composition. Productions of indoles or *p*-cresol dominantly correlate with protein intake from the diet [25]. Especially, undigested protein from the stomach strongly increases indoles and *p*-cresol in urine *via* increment of metabolism by intestinal flora [26,27]. Furthermore, a high-fat and high-purine body diet increases division of intestinal epithelial cells and peeled epithelial cells provide tryptophan to intestinal flora to increase skatole, indole-derivative, formation [28,29]. All of the above indicate that a "western-style," high-fat high-protein diet may dominantly increase the contents of intestinal toxins in feces. But until now, few studies have shown us the appropriate animal model of deteriorated enteral environment induced by a western-style diet.

In this paper, we aimed to establish a western-style-diet-induced deteriorated enteral environment animal model and investigated the effect of Agarwood (*Aquilaria crassna*) on intestinal toxins produced by an established high-fat and high-protein diet.

## Materials and Methods

### Materials

Agarwood (*Aquilaria crassna*) leaves were supplied by API Co., Ltd. (Gifu, Japan). PBS, *p*-dimethylaminobenzaldehyde, ammonium chloride, and indole were purchased from Wako Pure Chemical Co., Ltd. (Osaka, Japan). Manganese sulfate, trisodium phosphate, trisodium citrate, and sulfuric acid were purchased from Nacarai Tesque Inc. (Kyoto, Japan). Ethanol, sodium hypochlorite, and sodium hydroxide were from Kishida Chemical Co. Ltd. (Tokyo, Japan). Carmine was purchased from Sigma-Aldrich Corp. (St Louis, MO, U.S.A.). Food pellets (CE-2, CE-7, and Quick Fat) were purchased from CLEA Japan, Inc. (Tokyo, Japan).

### Extraction procedures of water extract of agarwood (WEA) and ethanol extract of agarwood (EEA)

WEA: Agarwood leaves (50 g) were chopped into small pieces and extracted with $H_2O$ at 95°C for 4 h. We got 8.0-9.0 g of solid powder from the leaves every time. EEA: Agarwood leaves (50 g) were chopped into small pieces and extracted with 60% ethanol (1000 ml) at room temperature (25.0°C) for 24 h. We got 8.0- 9.0 g of solid powder from leaves every time. The same lot as reference [30] is used in the present study.

### Animals

Male ddY mice (6 weeks old) were purchased from Japan SLC (Hamamatsu, Japan). The animals were housed at a controlled room temperature (24.5-25.0°C) with a 12/12 h light/dark cycle. Food pellets (CE-2, CE-7, Quick Fat) and tap water were provided *ad libitum*. Feeding regimen was shown at supplemental table 2. All animal experiments were carried out according to the "*Principles of Laboratory Animal Care*" (NIH publication number 85-23, revised 1985) and "*Guidelines of the Animal Investigation Committee of Gifu Pharmaceutical University*". All experiments were approved by the Animal Investigation Committee of Gifu Pharmaceutical University.

### Bacterial species and strains

*Escherichia coli* ATCC 25922, *Bacteroides vulgatus* GAI 0673, *Bacteroides fragilis* GAI 5524, *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* GAI 03013, *Clostridium difficile* GAI 93004, *Peponifhilus anaerobius* GAI 5506, *Bifidobacterium longum* GAI 91362, and *Bifidobacterium adolescentis* GAI 06099 were used for determination of MIC of WEA and EEA.

### Quantification of indoles in stool beads

Nine times weight of PBS was added to the stool beads (more than 50 mg) of mice and mixed very hard for 3-5 h until no lumps remain. The mixture was centrifuged at 5000 g for 15 min; this reagent is "stool samples in PBS." p-Dimethylaminobenzaldehyde was added to the "blank luminescence reagent" (5.2% (v/v) sulfuric acid / ethanol) at the rate of 1.47% (w/v); this regent is "the positive luminescence reagent." Fifty µl of "stool samples in PBS" were added to 300 µl of "the positive luminescence reagent" and "blank luminescence reagent" respectively, then mixed very hard and stilled at room temperature for 30 min. The mixture was centrifuged at 7500 rpm for 10 min and optical density at 568 nm was measured. Standard curve was made with indole preparation. The concentrations of indole derivatives were determined as $[(OD568_{\text{the positive luminescence reagent}} - OD568_{\text{blank luminescence reagent}})]$, compensating the matrix effect.

### Quantification of ammonium in stool beads

"Stool samples in PBS" for indole quantification was used for ammonium quantification. One hundred µl of *stool samples in PBS* and four hundred µl of PBS were put in the chilled centrifuging tube and gently mixed. Fifty µl of 3 mM manganese sulfate, 1 ml of phenol-alkali solution [6% (w/v) phenol, 3% (w/v) trisodium phosphate dodecahydrate, 3% (w/v)] trisodium citrate, and 0.3% (w/v) EDTA], and 500 µl of 0.12% available chlorine solution (sodium hypochlorite / 1M sodium hydroxide) were added to the chilled centrifuging tube. The tube was tightly sealed and boiled for 5 min mixing occasionally, then chilled in ice for 1 min, and kept at room temperature for 20 min, and then optical density at 635 nm was measured. Standard curve was made with ammonium chloride preparation.

### Measuring weight and number of stool beads

The wet weights and numbers of stools were measured as the frequency and total wet weight for each mouse over 8 h. WEA (1,000 mg/kg), EEA (1,000 mg/kg), or vehicle (10% gum arabic) was administered orally, and the frequency and weight of stools were measured for each mouse during consecutive 2-h periods (0-2 h, 2-4 h, 4-6 h, 6-8 h). The samples were prepared at a concentration of 100 mg/ml and administered 100 µl/10g. The mice were separated in small transparent cages (11 cm height, 17.5 cm width, and 11 cm depth, one mouse to a cage).

### Investigating the anti-microbial activities *in vitro* as Minimum Inhibitory Concentrations (MICs)

Minimum Inhibitory Concentrations (MICs) of Agarwood extracts were determined by broth dilution techniques, according to the instructions of the Clinical and Laboratory Standards Institute (CLSI). Broth MIC testing was performed in 96-well microtiter trays. Mueller–Hinton broth (MHB, Becton Dickinson, Cockeysville, MD, USA) was used for cultures of *Staphylococcus aureus* and *Escherichia coli*, Brain Heart Infusion broth (BHI, Nissui, Tokyo, Japan) was for *Enterococcus faecalis* and Reinforced Clostridial Medium (RCM, Oxoid LTD., Basingstoke, Hampshire, England) was for *Bacteroides vulgates, Bacteroides fragilis, Clostridium difficile, Peptostreptococcus anaerobius, Bifidobacterium longum, and Bifidobacterium adolescentis*. For the cultivation of *Bacteroides spp., C. difficile, P. anaerobius and Bifidobacterium spp.*, bacterial cultures were incubated under anaerobic condition in an anaerobic work-station, mini MACS (Don Whitley Scientific Led., West Yorkshire, England, UK). MIC values were obtained after incubation at 37°C for 24 h for cells of *S. aureus, E. coli* and *Ent. faecalis* or for 48 h for cells of *Bacteroides spp., C. difficile, P. anaerobius* and *Bifidobacterium spp.*. WEA and EEA were dissolved in 70% ethanol before addition to MHB, BHI and RCM for MIC determinations.

### Statistical analysis

Data are presented as mean ± S.E.M. Statistical comparisons were made with the Student's *t*-test, the one-way ANOVA with Tukey's multiple comparison test, or two-way repeated measure analysis of variance (ANOVA) with *t*-test (JSTAT for Windows; Vector, Tokyo, Japan).

## Results

### Effect of nutritional composition on body weights and stool parameters

We first investigated the effect of nutritional composition on body weight, wet weight of stools, and frequency of stools. We kept mice with three types of diet, CE-7 (normal diet), CE-2 (high-protein normal diet), and Quick Fat (high-protein high-fat diet). CE-2 and Quick Fat contain 1.4 times more protein than CE-7, and Quick Fat contains 3.7 times more fat than CE-7 (Supplemental table 1). There were no major differences between the diets in contents of moisture, crude fiber, crude ash, and nitrogen-free extract. We recognized no difference between the above three groups in body weights of mice, wet weights of feces, and number of fecal beads (Supplemental Figure 1).

### Effect of nutritional composition on ammonium and indoles in stool beads

We measured the contents of ammonium and indoles in fecal beads of the mice under regimens of three different diets (Supplemental Table 2A). As compared with CE-7, feces of mice under a regimen of CE-2 at the 8$^{th}$ day showed a tendency to increase in the contents of ammonium (from 1068 ± 330, n= 6, to 1513 ± 190 μg/g, n = 6) and indoles (from 25.1 ± 2.5, n = 6, to 28.3 ± 1.6 mg/g, n = 6) though not significant, and feces of mice under a regimen of Quick Fat showed a tendency to increase in ammonium contents (to 2101 ± 414 μg/g, n = 6) and showed significant increases in contents of indoles (to 51.7 ± 3.2 mg/g, n = 6) (Figure 1A and 1B). As compared with CE-7, feces of mice under regimen of CE-2 at the 14$^{th}$ day showed a tendency to increase in contents of ammonium (from 1045 ± 57, n = 6, to 1347 ± 184 μg/g, n = 6) and indoles (from 23.8 ± 2.1, n = 6, to 33.2 ± 2.4 mg/g, n = 6) though not significant, and feces of Quick Fat-fed mice showed significant increases in contents of ammonium (to 3614 ± 659 μg/g, n = 6) and indoles (to 49.1 ± 5.3 mg/g, n = 6) (Figure 2A and 2B).

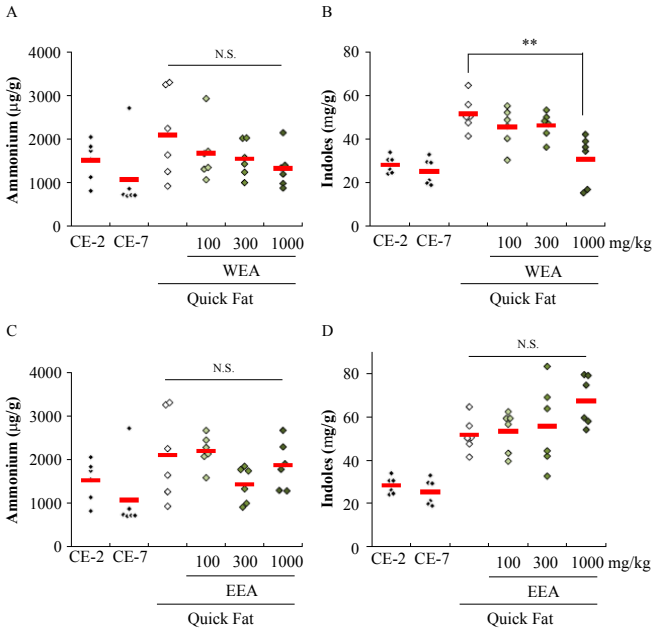


**Figure 1: Effects of single administrations of WEA and EEA on mice under regimen of Quick Fat.** Mice received a regimen of CE-2, CE-7, or Quick Fat. Then, WEA and EEA were orally administered to the mice under a regimen of Quick Fat at 100, 300, and 1,000 mg/kg according to the schedules shown in Supplemental Table 2B. We collected fresh fecal beads of the mice over 50 mg at 8 h after the first administration of the samples and determined the contents of ammonium and indoles; (A, C): contents of ammonium, (B, D): contents of indoles. Determinations of indoles were executed on the same day as the collection of feces or the next day.

Average scores of each group were shown as square. n = 6, **: $p < 0.01$ (one-way ANOVA and Tukey's multiple comparison test).

### Effects of WEA and EEA on ammonium and indoles in stool beads

We orally administered WEA and EEA at 100, 300, and 1,000 mg/kg once per day for 7 days to the mice under a regimen of Quick Fat (Supplemental Table 2B). We measured the contents of ammonium and indoles in fecal beads of the mice after single administration and 7 days of multiple administrations. Single administration of WEA showed a tendency to decrease the contents of ammonium in feces from 2101 ± 414 to 1673 ± 271, 1548 ± 170, and 1322 ± 184 μg/g, n = 6, at 100, 300, and 1000 mg/kg, n = 6, respectively, and WEA significantly decreased the contents of indoles in feces from 51.7 ± 3.2 to 30.7 ± 4.8 mg/g, n = 6, at 1,000 mg/kg (Figure 1A and 1B). Dose-dependent decrease of indole was seen at the lower dose (45.4 ± 3.7 and 46.3 ± 2.5 mg/g, n = 6, at 100 and 300, respectively). Single administration of EEA failed to affect the contents of ammonium and indoles in feces (Figure 1C and 1D). Seven days of multiple administrations of WEA dose-dependently showed a decrease of ammonium and indoles in feces (ammonium: from 3614 ± 659 to 3046 ± 636, 2906 ± 384, and 1429 ± 274 μg/g, n = 6; indole: from 49.1 ± 5.3 to 57.2 ± 4.8, 34.8 ± 5.8, and 25.6 ± 4.4 mg/g, n = 6) to the same level as those of CE-7 (ammonium: 1045 ± 57 μg/g, n = 6; indole: 23.8 ± 2.1 mg/g, n = 6) (Figure 2A and 2B). Multiple administrations of EEA did not affect the contents of ammonium in feces, though the

contents of indoles decreased dose-dependently and significantly at 1,000 mg/kg (from 49.1 ± 5.3 to 24.4 ± 1.1 mg/g, n = 6).

### The effects of WEA and EEA on body weight and stool parameters

We examined the body weight and wet weight and number of stools after 7 days of multiple administrations of the samples. WEA and EEA did not affect body weight at any dose and did not affect wet weight or number of stools at 1,000 mg/kg (Supplemental Figure 2).

### The effects of WEA and EEA on carmine egestion

We examined carmine egestion to investigate activities of the digestive tract, which has a strong effect on intestinal toxin production. Carmine egestion was monitored every hour for 8 h after oral administration of carmine (10 mg/body). Carmine egestion is defined as the ingestion of carmine-containing feces over 30 mg cumulatively. As compared with CE-7, feeding Quick Fat dominantly delayed the carmine egestion, though not significantly, and administrations of WEA and EEA at 1,000 mg/kg significantly accelerated the carmine egestion (Table 1).

| Treatments | mg/kg | $x^2$ test vs. Quick Fat | Scores | | |
|---|---|---|---|---|---|
| | | | Fast (2–5 h) | Medium (5–8 h) | Slow (over 8 h) |
| CE-7 | | p = 0.148 | 3 | 4 | 1 |
| Quick Fat | | | 0 | 7 | 1 |
| WEA (Quick Fat) | 1000 | p = 0.022* | 5 | 3 | 0 |
| EEA (Quick Fat) | 1000 | p = 0.008** | 6 | 2 | 0 |
| WEA: water extract of agarwood | | | | | |
| EEA: ethanol extract of agarwood | | | | | |

Mice received a regimen of CE-7 or Quick Fat for a week. A vehicle (10% gum arabic), WEA, and EEA at 1,000 mg/kg were orally administered to the mice. Carmine (10 mg/body) was orally administered to the mice just before administration of the samples, and then the weights of carmine-containing feces were measured during consecutive 1-h periods over 8 h. Carmine egestion was determined as egestion of carmine-containing fecal beads over 30 mg. The carmine egestion point was classified into three groups; Fast: egested in 2–5 h, Medium: egested in 5–8 h, Slow: not egested in 8 h after administration n = 8.

**Table 1:** Effects of WEA and EEA on carmine egestion in mice.

### The effects of WEA and EEA administration interruption on decreased intestinal toxin

After 7 days of multiple administrations of WEA and EEA, we aborted the administration and kept the mice for 7 days, and then we measured the contents of ammonium and indoles in fecal beads again. The significantly decreased contents of ammonium and indoles in fecal beads of the mice under daily administration of WEA (ammonium: 1937 ± 244, 1602 ± 362, and 2209 ± 326 μg/g, n = 6; indoles: 33.9 ± 2.4, 33.1 ± 1.3, and 39.2 ± 4.2 mg/g, n = 6; at 100, 300, and 1,000 mg/kg) were increased to the same levels as the vehicle-administered mice (ammonium: 2010 ± 85 μg/g, n = 6; indoles: 39.4 ± 1.7 mg/g, n = 6) after interruption of administration (Figure 3A and B). The significantly decreased contents of the indoles of mice under daily administration of EEA (45.7 ± 2.6, 40.2 ± 1.7, and 36.0 ± 1.9 mg/g; at 100, 300, and 1,000 mg/kg, n = 6) were also increased to a level of the vehicle-administered mice (39.4 ± 1.7 mg/g, n = 6) after interruption of administration (Figure 3D).

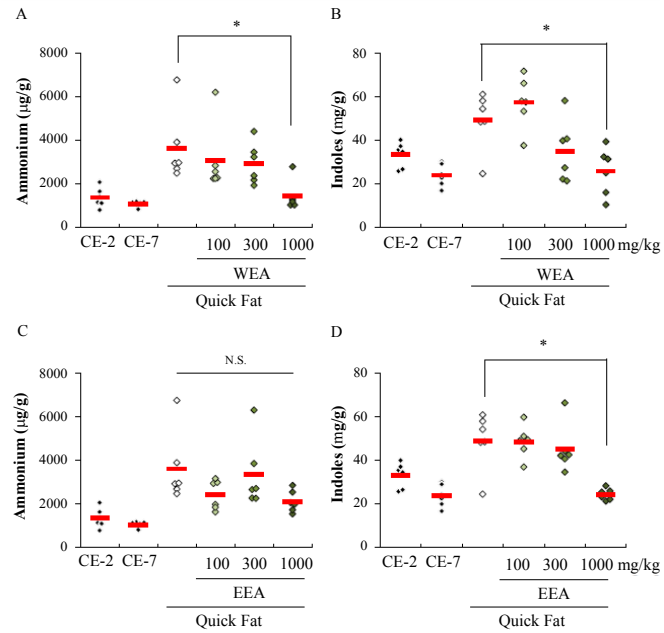


**Figure 2: Effects of multiple administrations of WEA and EEA on mice under a regimen of Quick Fat.** The experiments were performed as a continuation of Figure 1. We collected fresh fecal beads of the mice over 50 mg at 8 h after the seventh administration of the samples and determined the contents of ammonium and indoles; (A, C): contents of ammonium, (B, D): contents of indoles. Determinations of indoles were executed on the same day as the collection of feces or the next day.

Average scores of each group were shown as a square. n = 6, *: $p < 0.05$ (one-way ANOVA and Tukey's multiple comparison test)

### The antimicrobial activities of WEA and EEA *in vitro* as Minimum Inhibitory Concentrations (MICs)

Both WEA and EEA showed significant antimicrobial activities against gram-positive and gram-negative bacteria, such as *Bacteroides vulgas* (MIC = 8 mg/ml), Bacteroides longum (MIC = 8 mg/ml), *Staphylococcus aureus* (MIC = 4), *Clostridium difficile* (MIC = 8 and 4 mg/ml [WEA and EEA, respectively]), and *Peptostreptococcus anaerobius* (MIC = 4 mg/ml) (Table 2). On the other hand, neither WEA nor EEA showed antimicrobial activities against *Eecherichia coli*, *Enterococcus faecalis,* or *Bifidobacterium spp.* (MICs > 8 mg/ml). Similar MIC values were obtained in two independent experiments performed in duplicate.

## Discussion

In the present study, we first verified that Quick Fat, a commercial diet developed to research type 2 diabetes, deteriorates the enteral environment, as does a western-style diet. A regimen of Quick Fat ordinarily takes more than 14 weeks to induce type 2 diabetes, though blood cholesterol increases at the early stage, less than 15 days, of a regimen [31]. In contrast, our study shows that a regimen of Quick Fat over only 1 week significantly increased the contents of indoles as compared with CE-7 (Figure 1B, 2B, and 3B), while the contents of ammonium significantly increased with a regimen of Quick Fat over 2 weeks (Figure 2B and 3B). We kept mice with Quick Fat for 3 weeks, and then we recognized that a regimen of Quick Fat for only 3 weeks did not affect body weight, indicating that no mice are obese (Supplemental Figure 1A). A four weeks' regimen of Quick Fat did not affect wet weight or number of stools, indicating that no mice are suffered from constipation (Supplemental Figures 1B and 1C). A regimen of CE-2

over 1 week, high-protein diet, showed a tendency to increase the contents of ammonium and indoles as compared with CE-7 (Figures 1, 2, and 3), but not significantly. All of the above indicate that high-protein and high-fat contents act additively to increase the contents of ammonium and indoles in fecal beads of mice. A regimen of high-fat diet grows *Clostridium perfringens*, a bacterial strain that elaborates both the indoles and ammonium, particularly when sodium cholate, an ionic-emulsifying agent secreted into the bile from the liver, is added [32]. It makes sense that protein contained in the diet provides the basic ingredients of ammonium and indoles; on the other hand, fat in the diet grows ammonium- or indole-positive germs in the gastrointestinal tract. From these points of view, we judged that a regimen of Quick Fat as compared with CE-7 is useful animal model for a deteriorated enteral environment model, which is available for research of intestinal toxins apart from constipation.

We administered WEA and EEA at 100, 300, and 1,000 mg/kg once per day for 7 days to the mice under a regimen of Quick Fat. A single administration of WEA at 1,000 mg/kg has significantly decreased indoles, but not EEA (Figure 1B and 1D). On the other hand, single administrations of WEA and EEA failed to decrease ammonium significantly, though WEA showed such a tendency (Figure 1A and 1C). Single administrations of WEA and EEA significantly accelerated carmine egestion under a regimen of Quick Fat (Table 1). The quantities of intestinal putrefactions are estimated to bear a proportionate relationship between total retention times of contents in gastrointestinal tract. But the failure of EEA to decrease ammonium and indoles is unexplainable *via* acute acceleration of the gastrointestinal contents.

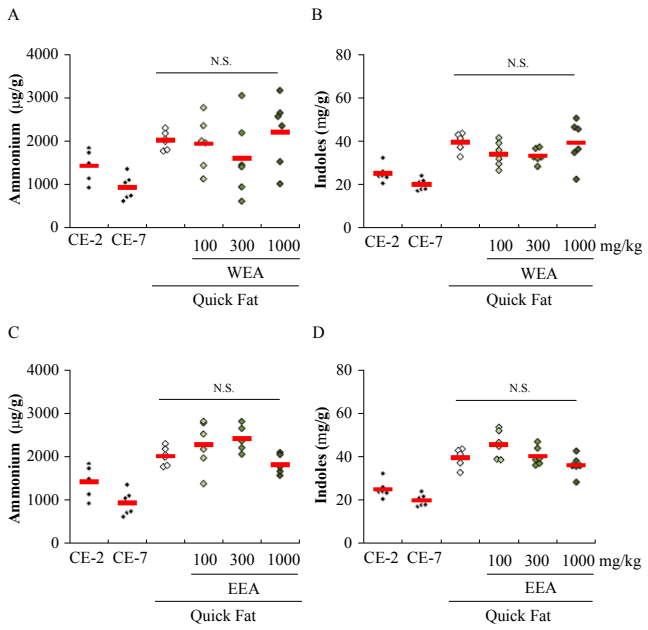


**Figure 3: Effect of seven days-interruption of WEA and EEA administration on mice under a regimen of Quick Fat.** The experiments were performed as a continuation of Figure 2. We aborted administration of the samples in all groups after the seventh administration, and then we collected feces of the mice over 50 mg one week later and determined the contents of ammonium and indoles; (A, C): contents of ammonium, (B, D): contents of indoles. Determinations of indoles were executed on the same day as the collection of feces or the next day.

Average scores of each group were shown as a square. n = 6, N.S.: $p > 0.05$ (one-way ANOVA and Tukey's multiple comparison test).

| | MICs of Agarwood extracts (mg/ml) | | |
|---|---|---|---|
| | WEA | EEA | Ammoniagenesis* |
| G (-) Bacteria | | | |
| *Escherichia coli* | > 8 | > 8 | + [37,38] |
| *Bacteroides vulgatus* | 8 | 8 | + [38] |
| *Bacteroides fragilis* | 8 | 8 | + [38] |
| G (+) Bacteria | | | |
| *Staphylococcus aureus* | 4 | 4 | + + [37] |
| *Clostoridium difficile* | 8 | 4 | + [38] |
| *Peptostreptococcus anaerobius* | 4 | 4 | + + [39-41] |
| *Enterococcus faecalis* | > 8 | > 8 | + [42] |
| *Bifidobacterium longtum* | > 8 | > 8 | – [39] |
| *Bifidobacterium adolescentis* | > 8 | > 8 | – [39] |

*Ammoniagenesis was graded according to the previous reports; ++: strong positive, +: positive, –: negative

**Table 2:** Minimum inhibitory concentrations (MICs) of WEA and EEA.

Multiple administrations of WEA at 1,000 mg/kg significantly decreased the contents of ammonium and indoles (Figure 2A and 2B). On the other hand, EEA at 1,000 mg/kg significantly decreased the contents of indoles, but not ammonium (Figure 2C and 2D). Multiple administrations of WEA and EEA did not affect the wet weight of stools or number of stools (Supplemental Figure 2B and 2C), indicating that ammonium and indole concentrations, mg/g, in fecal beads are proportional to their production quantities, g, in the gastrointestinal tract of a mouse. Considering the result that single administrations of WEA and EEA did not affect the contents of ammonium, ammonium might be insusceptible to gastrointestinal transit time and be influenced by another predominant factor, such as microbial environment.

We then investigated the antimicrobial activities of WEA and EEA against urease-positive and -negative enterobacteria. Both WEA and EEA showed antimicrobial activities on some urease-positive enterobacteria at 4 to 8 mg/ml (Table 2), at lower concentrations than the administration solution in the animal experiments (10 to 100 mg/ml) we presented at Figures 2 and 3. On the other hand, WEA or EEA did not affect *Escherichia coli* and lactic acid bacteria (*Enterococcus faecalis*, *Bifidobacterium longum*, and *Bifidobacterium adolescentis*). Previous report shows that some tea polyphenol show bactericidal activities on *Staphylococcus aureus* and *Clostridium perfringens* but not on *Escherichia coli* and *Bifidobacteria* [33]. On the other hand, Escherichia coli and Bifidobacteria is susceptible to pH of gastrointestinal tract [34], not to botanical polyphenols. The difference of susceptibility to antibacterial effect of WEA and EEA is depending on susceptibility to botanical polyphenols. Interestingly, among the nine strains we examined in the present study, only *Enterococcus faecalis* and *Escherichia coli* are indole-producing bacteria [35,36], and neither strain is susceptible to the antimicrobial activities of WEA or EEA (Table 2). Urease, an ammonia genesis enzyme, is active mainly at the mucosa of the small intestine (mammalian-derived urease) and large bowel flora (bacteria-derived urease), meaning that a half of ammonium production in the intestine belongs to the metabolism of mammalians. Indole production in the intestine depends mainly on indole-positive bacteria. Difference of susceptibility to WEA between indoles and ammonium may depend on the producing enterobacteria

and derivation mechanisms. These findings indicate that WEA may interfere with the total span of indole production *via* shortening the retention time of gastrointestinal contents at a short span, and interfere with the rate of ammonium production *via* antimicrobial activities against urease-positive enterobacteria.

At the end of a set of regimens, we aborted the administrations of WEA and EEA to verify the hypothesis stating the decrement of indole or ammonium requires continuous administration. A one-week cessation of administration abolished the decrement of indole and ammonium (Figure 3), meaning that the decreased production quantities during the administration regimens increased to the same level as the vehicle groups after cessation of administration. This result indicates that we need continuous administrations of WEA or EEA to remedy the enteral environment deteriorated by a western-style diet.

In summary, a comparison between CE-7 and Quick Fat is useful to research western-style-diet-induced deteriorated enteral environments. Quick Fat increased the concentrations of ammonium and indoles in fecal beads, which is proportional to production quantities, without inducing constipation or increment of body weight. Single administrations of WEA and EEA significantly accelerated carmine egestion. Single administrations of WEA significantly decreased the production quantities of indoles in gastrointestinal tract and multiple administrations of WEA significantly decreased the production quantities of ammonium and indoles. WEA and EEA showed significant antimicrobial activities against some urease-positive enterobacteria, but not indole-positive enterobacteria. The series of decrements of intestinal putrefaction require continuous administrations.

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