Influence of Dried Okara-Tempeh on the Composition and Metabolites of Fecal Microbiota in Dogs

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ABSTRACT

This study investigated the influence of dried okara-tempeh, the insoluble portion of soybeans fermented with ragi-tempeh, on fecal microbiota and metabolites in dogs. Three dried okara-tempehs (individual weight: approximately 5 g) were given daily to each of 6 beagle dogs for a period of 2 weeks. The concentrations of fecal short-chain fatty acids, pH, and microbiota were determined. During the intake of the dried okara-tempeh, the level of *Bifidobacterium* expressed as $\log_{10}$ number (CFU)/g wet feces was increased significantly ($p < 0.05$) from 6.4 ± 0.9 (mean ± SD) to 8.8 ± 0.8, and the level of *Bacillus* was also increased significantly ($p < 0.05$) from 4.1 ± 1.8 to 7.3 ± 0.6. Moreover, fecal concentrations of total short-chain fatty acids, acetic acid and propionic acid also increased significantly ($p < 0.05$) from 146.7 ±15.9 μmol/g wet feces to 198.8 ± 24.6μmol/g wet feces, from 90.3 ±8.6 μmol/g wet feces to 116.3 ± 17.9μmol/g wet feces, and from 39.3 ±3.7 μmol/g wet feces to 60.0 ± 9.1μmol/g wet feces respectively on day 7 of dried okara-tempeh intake compared to pre-intake. Fecal pH decreased significantly ($p < 0.05$) from 6.2 ± 0.3 to 5.6 ± 0.2 during intake. The findings obtained by this study demonstrate that the intake of dried okara-tempeh was effective for improving the fecal environment in dogs and that okara, which is currently

discarded, can be effectively utilized in dog feed.

INTRODUCTION

Okara is a white or yellowish pulp consisting of the insoluble portion of soybeans. It is low in fat, high in fiber and contains protein, calcium, iron, and riboflavin. In Japan, okara is mainly obtained when soy milk, which is used as the starting material for several foods, such as tofu, is obtained from soybeans. In Japan, okara is used as a food and livestock feed, or discarded.

Soybeans contain oligosaccharides, which act as a growth promoter for bifidobacteria. It has been reported that prebiotics such as oligosaccharides and dietary fiber improve the balance of the intestinal microbiota by enhancing the growth of beneficial intestinal bacteria, such as bifidobacteria, and/or inhibiting the growth of harmful bacteria. This results in scavenging in the intestinal environment. The maintenance of an optimal balance of intestinal microbiota is achieved by the intake of prebiotics.[1] Therefore, it is thought that the intake of okara is related to improvements in the intestinal environment.

It is known that diet affects the composition of intestinal microbiota and the predominance of harmful bacteria such as clostridia in the intestines is related to various disturbances.[1] Harmful bacteria may produce putrefactive products such as ammonia, hydrogen sulfide, amines, phenols and indoles. These products potentially contribute to host diseases[1] and are also the cause of fecal odor.[2] Terada et al[2,3] reported that an improvement of intestinal microbiota and the intestinal environment was associated with a deodorant effect. Kimura et al.[4] reported that the administration of a bifidobacteria preparation seemed to reinforce the recovery of the normal intestinal microbiota and alleviate clinical symptoms in scouring animals. Therefore, it is thought that improvement of the intestinal microbiota is related to host health and decreased fecal odor. For pet animals, fecal odor and diarrhea caused by the diet can be a serious problem.

Tempeh is a traditional fermented Indonesian food in which fungi, particularly *Rhizopus* spp., play an essential role. There are many reports on the beneficial effects of tempeh.[5-7] It has also been suggested that the extract of soybean-tempeh inhibit the adhesion of enterotoxigenic *Escherichia coli* K88 to the small intestinal brush-border in piglets.[8] Since large amounts of okara are discarded in Japan, its use in animal feed would be an efficient application of an underutilized resource.

In the present study, an investigation of the effect of dried okara-tempeh intake on fecal microbiota and metabolites was carried out using beagle dogs.

MATERIALS AND METHODS

Preparation of Dried Okara-Tempeh

Dried okara-tempeh (Okabeya Co., Ltd., Kanagawa, Japan) was prepared as follows. Vinegar was added to freshly harvested okara (temperature of okara was approximately 90°C) and the mixture was cooled rapidly to 20°C. Cooled okara was inoculated with ragi tempeh (tempeh culture; Aneka Fermentation Industry, Bandung, Indonesia). The mixture was wrapped, and incubated at 30°C for 24 hours. After incubation, the fermented okara was placed in a gas oven at 120°C for 10 minutes to stop the fermentation and left in the gas oven at 65°C for 8 hours to continue drying.

Animals

The animals used in this study were 6 adult beagles (all females), weighing 8.6 to 11.4 kg (mean±SD, 9.9±1.1). They were bred at Nippon Veterinary and Life Science University, Musashino-shi, Tokyo. The dogs were maintained under standardized environmental conditions (room temperature, 20-26°C; relative humidity, 40-70%). During the experiment, the 6 dogs were housed individually in cages with a wire-mesh floor through which feces fell onto a stainless steel tray. The dogs used in this experiment were treated in accordance with Nippon Veterinary and Life Science University's ethical guidelines for animal care and handling.

Table 1. *Compositions of the commercial diet (Vets Plan pH-care) used in this study*

Ingredients	/100 g	Ingredients	/100g
Protein	25.0 g	Taurine	0.3 g
Fat	16.0 g	Arginine	1.6 g
Fiber	6.7 g	Vitamin A	3280 UI
Ash	7.3 g	Vitamin E	60.0 mg
Water	8.0 g	Vitamin B_2	5.5 mg
Carbohydrate	42.2 g	Vitamin C	20.0 mg
Ca	0.9 g	Vitamin D_3	81 UI
K	0.6 g	Vitamin B_1	5.5 mg
P	0.7 g	Vitamin B_2	5.5 mg
Mg	0.1 g	Pantothenic acid	16.6 mg
Fe	18.0 mg	Vitamin B_6	8.7 mg
Cu	2.2 mg	Vitamin B_{12}	0.02 mg
Zn	24.5 mg	Nicotinic acid	50.0 mg
Cl	1.1 g	Biotin	0.3 mg
Na	0.9 g	Folic acid	1.8 mg
Se	0.02 mg	Corrin	150.0 mg
EPA+DHA	300.0 mg		396 kcal
L-carnitine	-		

Experimental Design and Diets

A total of 280 g of a commercial dog diet (Vets Plan pH-care, Royal Canine, Japan, Inc., Tokyo, Japan), as shown in Table 1, was given per day to each animal for 1 week before the dried okara-tempeh intake period. This intake period lasted for 2 consecutive weeks, during which 3 dried, stick-shaped okara-tempehs (individual weight: approximately 5 g) were given per day to each animal, in addition to the commercial dog diet described above. A commercial diet was then fed for 1 additional week. Water was available *ad libitum* during the experimental period, provided in a stainless dog bowl. The concentrations of fecal short-chain fatty acids, facal pH and fecal microbiota were determined on sampling days 0, 7, 14, as well as 7 days after the final intake.

Fecal Measurements

Fecal samples collected from each dog were immediately analyzed for fecal microbiota and pH. The remainder of the samples was frozen at −80°C for the later analysis of bacterial metabolites. The fecal microbial analysis was carried out employing the methods of Mitsuoka et al.[9,10] and Hara et al.,[11] as described previously.[12] After thorough mixing, a series of 10-fold dilutions (10^{-1} to 10^{-8}) were made in anaerobic diluents. From the appropriate diluents, 0.05 mL aliquots were spread onto 3 non-selective agars: modified Eggerth-Gagnon (EG) agar for anaerobes, glucose-blood-liver (BL) agar for anaerobes, and trypticase soy blood (TS) agar (Becton, Dickinson and Company, Sparks, MD, USA) for aerobes. In addition, 0.05 mL aliquots were spread onto 11 selective agars: bifidobacteria selective (BS) agar for bifidobacteria, eubacteria selective (ES) agar for Gram-positive anaerobic rods such as *Eubacterium*, neomycin-brilliant green-taurocholate-blood (NBGT) agar for bacteroides, neomycin-Nagler (NN) agar for lecithinase-positive clostridia, modified

Table 2. *Effect of dried okara-tempeh intake on fecal microbiota in 6 dogs*[a]

Organism	Before intake	During intake		After intake
	Day 0	Day 7	Day 14	Day 7
Total bacteria	10.4±0.2	10.5±0.1	10.5±0.2	10.6±0.2
Bifidobacterium	6.4±0.9 (67)	8.8±0.8* (83)	8.5±0.9* (100)	7.9±1.9 (100)
Bacteroidaceae	10.1±0.2 (100)	10.0±0.2 (100)	10.2±0.3 (100)	10.3±0.1 (100)
Anaerobic non-spore-forming Gram positive rods	9.4±0.4 (100)	9.7±0.4 (100)	9.8±0.3 (100)	9.9±0.2 (100)
Anaerobic Gram positive cocci	8.9±0.5 (83)	9.4±0.2 (100)	9.4±0.4 (100)	9.5±0.7(100)
Lecithinase-positive *Clostridium*	4.5±2.0 (50)	3.6 (33)	(0)	3.6±1.6 (67)
Lecithinase-negative *Clostridium*	7.5±0.8 (67)	7.2±1.5 (83)	8.4±0.8 (83)	8.8±0.9 (67)
Lactobacillus	8.5±1.8 (100)	9.2±0.9 (88)	8.4±1.3 (100)	8.4±1.4 (100)
Enterobacteriaceae	6.6±1.1 (100)	6.7±0.8 (100)	6.4±1.0 (100)	6.4±1.3 (100)
Streptococcus and *Enterococcus*	9.0±1.0 (100)	8.9±0.8 (100)	8.4±1.0 (100)	8.9±0.7 (100)
Staphylococcus	3.2±1.0 (83)	4.0±1.1 (50)	4.4±0.3 (67)	4.2±0.9 (100)
Bacillus	4.1±1.8 (50)	7.3±0.6*, ** (100)	6.7±0.8* (100)	6.2±0.6 (50)
Pseudomonas	4.5±0.6 (83)	3.8±1.1 (100)	3.9±0.8 (67)	3.7±0.4 (50)
Yeasts	(0)	(0)	(0)	5.1 (17)

[a]*Values are expressed as the mean of the $\log_{10}$ number±SD (CFU)/g wet feces. Figures in parentheses are frequency of occurrence (%).*

**Significant difference from the value on day 0 (before intake) at $p < 0.05$.*

***Significant difference from the value on day 7 (after intake) at $p < 0.05$.*

veillonella selective (VS) agar for veillonellae and megasphaerae, modified lactobacilli selective (LBS) agar for lactobacilli, triphenyltetrazolium chloride-acridine orange-thallous sulfate-esculin-crystal violet (TATAC) agar for enterococci and streptococci, *Staphylococcus* medium no. 110 agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) with phenylethyl alcohol egg-yolk suspension (PEES) agar for staphylococci, potato dextrose (PD) agar (Nissui) for yeasts and molds, DHL agar (Nissui) for *Enterobacteriaceae* and NAC agar (Nissui) for *Pseudomonas aeruginosa*. Moreover, TOS propionate agar (Yakult Pharmaceutical lnd., Co., Ltd., Tokyo, Japan) was also used for bifidobacteria. Nine agars (EG, BL, NBGT, BS, ES, NN, VS, LBS, and TOS propionate agars) were incubated at 37°C for 3 days in an anaerobic steel wool jar filled with an atmosphere of oxygen-free CO_2. Four agars (TATAC, PEES, NAC, and PD) were incubated aerobically at 37°C for 48 hours, and TS and DHL agars were incubated at 37°C for 24 hours. In addition, dilutions (10^{-1}, 10^{-3}, and 10^{-5}) of the fecal specimens were heated at 80°C for 10 minutes to select clostridial spores and a portion of 0.05 mL of each dilution was used to inoculate CW agar (Nissui) containing egg-yolk emulsion, and incubated anaerobically at 37°C for 3 days using an AnaeroPack-Kenki (Mitsubishi Gas, Tokyo, Japan). After incubation, each plate was examined for bacterial colonies. The identification of bacterial groups and yeasts was performed based on Gram reaction, colonial and cellular morphology, spore formation, aerobic growth

and selected biochemical characteristics. The results are expressed as the $\log_{10}$ of the number of bacteria (CFU)/g wet weight of fecal material. Fecal concentrations of short-chain fatty acids were analyzed employing a high-performance liquid chromatography organic acid analysis system (HPLCOA, Shimadzu Co., Ltd., Kyoto, Japan) using the method of Hara et al.[11] Fecal pH values were determined as described previously.[12]

Statistical Analysis

Student's *t*-test was used for statistical analysis of the fecal pH, microbiota, and short-chain fatty acids. A probability value of $p < 0.05$ was considered significant.

RESULTS

There were no clinically abnormal events in any dogs throughout the experimental period.

The results of fecal microbiota analysis are shown in Table 2. During the intake of dried okara-tempeh, the numbers of *Bifidobacterium* and *Bacillus* increased significantly ($p < 0.05$) when compared to the values before intake. No significant changes in the numbers of other intestinal bacteria during the experimental period were observed.

A significant increase ($p < 0.05$) in the fecal concentration of short-chain fatty acids, such as total acids and acetic acid, was observed during the dried okara-tempeh intake period. The concentration of propionic acid also increased significantly ($p < 0.05$) when compared to the value before intake, as shown in Table 3. Furthermore, the fecal pH showed a significant decrease ($p < 0.05$) with dried okara-tempeh intake (Table 3).

Table 3. *Effect of dried okara-tempeh intake on fecal properties in 6 dogs*[a]

Item	Before intake	During intake		After intake
	Day 0	Day 7	Day 14	Day 7
pH	6.2±0.3	5.6±0.2*,**	5.6±0.2*,**	6.3±0.4
Short-chain fatty acids				
Total acids (μmol/g wet feces)	146.7±15.9	198.8±24.6*, **	188.0±16.6*, **	147.5±31.5
Acetic acid (μmol/g wet feces)	90.3±8.6	116.3±17.9*, **	106.0±8.3*, **	86.2±16.4
Propionic acid (μmol/g wet feces)	39.3±3.7	60.0±9.1*	58.4±8.4*	44.0±13.8
Butyric acid (μmol/g wet feces)	11.5±4.6	15.9±4.1	18.7±9.2	11.3±2.7
iso-Butyric acid (μmol/g wet feces)	2.6±0.6	2.4±0.8	1.9±0.7	2.4±0.3
Formic acid (μmol/g wet feces)	0.5±0.2	0.5±0.2	0.3±0.1	0.4±0.0
Valeric acid (μmol/g wet feces)	Not detected	3.1	2.3	6.5
iso-Valeric acid (μmol/g wet feces)	2.7±0.8	2.6±0.6	2.1±1.0	2.4±1.0

[a]*Values are expressed as mean±SD.*
**Significant difference from the value on day 0 (before intake) at $p < 0.05$.*
***Significant difference from the value on day 7 (after intake) at $p < 0.05$.*

DISCUSSION

Since most okara in Japan is discarded, finding a use for it, such as a food, is desirable. However, okara is hard to digest, so the improvement of its digestibility is necessary for the development of a favourable food. Okara fermented with *Rhizopus* contains more free amino acids, acid-soluble nitrogen, free sugars and inorganic phosphorus, and less fiber than a non-fermented control.[13] These findings show that several ingredients of okara are broken down into low-molecular-weight materials and digestibility is improved upon fermentation with *Rhizopus*. Furthermore, the antioxidant activity of okara-tempeh is higher than that of okara.[13] As a result of these reports, the development and utilization of okara-tempeh has been advancing recently. On the other hand, it has also been suggested that an extract of soybean-tempeh inhibits the adhesion of enterotoxigenic *E. coli* K88 to the small intestinal brush-border of piglets[8]. Diarrheagenic *E. coli* is associated with intestinal diseases such as diarrhea in humans and animals. Enterotoxigenic *E. coli* has also been isolated from dogs.[14] It has been reported that pets can be natural reservoirs of several organisms potentially able to cause disease in humans.[15] For these reasons, the use of okara-tempeh as a food for dogs was investigated in the present study.

The intestinal microbiota is known to play an important role in host health.[1,16] It has also been reported that bifidobacteria and lactobacilli, which are members of the intestinal microbiota, produce lactic acid and do not produce putrefactive products, and affect the host by improving the intestinal bacterial balance.[1]

Our results show that the number of *Bifidobacterium* increased significantly with dried okara-tempeh intake. It has been reported that the changes seen in populations of lactobacilli, bifidobacteria, and *Enterobacteriaceae* in the lower intestine may be closely related to the clinical symptoms.[4] An increase in Enterobacteriaceae and a decrease in bifidobacteria and lactobacilli were noted in scouring dogs.[17] Based on the results of the present study, the oral administration of dried okara-tempeh helps in the normalization of the intestinal microbiota.

It was reported that the intake of soybean oligosaccharides is related to increased fecal bifidobacteria in humans.[18] It has also been reported that soybean oligosaccharides are used selectively by bifidobacteria and are not fermented by *Clostridium perfringens* and *E. coli*.[11,18] Similar results were reported in fermentation tests of purified stachyose and raffinose fractions of soybean oligosaccharides.[18] These findings indicate that soybean oligosaccharides are one of the factors involved in increasing intestinal bifidobacteria. Benno et al.[19] reported a significant increase in human fecal bifidobacteria brought about by the intake of raffinose. The present study revealed that the intake of dried okara-tempeh resulted in a decrease in the fecal pH as well as an increase in fecal short-chain fatty acids, such as acetic acid, and the number of fecal *Bifidobacterium*. The increasing number of *Bifidobacterium* and concentration of short-chain fatty acids brought about by the dried okara-tempeh intake may result in the lower pH levels of feces. These findings suggest that carbohydrates such as oligosaccharides in dried okara-tempeh are one of the substrates involved with increasing intestinal bifidobacteria. Terada et al.[2] described that the decreased fecal pH results from the increased levels of bifidobacteria which leads to a reduction of the production of ammonia. Putrefactive products are the cause of fecal odor.[2] Hayakawa et al.[18] reported that the amount of fecal indole shows a negative correlation with the number of fecal *Bifodobacterium*. In the present study, the offensive odor of feces decreased during the intake of the dried okara-tempeh. Owing to the decrease in the fecal pH and the increase of the number of fecal *Bifidobacterium*, it is thought that the fecal concentration of putrefactive products decreased. In order to investigate this, it is necessary to measure the amount of soybean oligosaccharides in dried okara-tempeh.

During the intake of dried okara-tempeh, the number of *Bacillus* also significantly increased. It has been reported that *Bacillus* was isolated from commercial tempeh[20] and tempeh during fermentation.[21,22] *Bacillus pumilus* and *Bacillus brevis* are the predominant bacterial species, achieving populations of 10^8 CFU/g during the fungal fermentation of soybean-tempeh.[22] The predominance of *Bacillus* spp. in samples of freshly prepared tempeh was also reported.[23] *Bacillus* was also isolated from the dried okara-tempeh examined in this study. Therefore, an increase in *Bacillus* may be related to the intake of okara-tempeh. Some *Bacillus* species such as *Bacillus subtilis*,[24, 25] *Bacillus cereus*[26] and *Bacillus coagulans*[27] are used as probiotics for animals. In addition, the administration of *B. subtilis*[25] resulted in an increase in fecal bifidobacteria. On the other hand, it is well-known that *B. cereus* is a bacterium associated with food poisoning. *B. cereus* is usually present in soil, water, air and on plants. It was also isolated from commercial soybean-tempeh.[20] Since there were no abnormal findings in dogs throughout the experimental period, it is thought that the increase of fecal *Bacillus* is not harmful and *Bacillus* contained in dried okara-tempeh is not pathogenic in dogs. There have been no reports of food poisoning due to soybean-tempeh.[7]

The results of this study indicate that the intake of dried okara-tempeh is related to the maintenance of the optimal balance of intestinal microbiota and a favorable intestinal environment. In addition, okara, which is currently discarded in many cases, can be effectively utilized in dog feed.

Further studies involving a larger number of animals are needed to confirm these results.

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