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Full Paper

Continuous intake of galacto-oligosaccharides containing syrup contributes to maintaining the health of household dogs by modulating their gut microbiota

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Interest is growing in the relationship of the microbiota and intestinal environment with health in companion animals. Galacto-oligosaccharides (GOS), typical prebiotics, are expected to provide benefits in dogs. Previous studies of GOS in dogs have involved dogs with similar rearing conditions and diets, which may have biased the results. We conducted an open study of 26 healthy dogs kept in households with diverse rearing environments in order to evaluate how the intake of a GOS-containing syrup affects the intestinal microbiota and its metabolites. Each dog was fed 1.2–4.8 g of the GOS-containing syrup (GOS 0.5–2.0 g equivalent) for 8 weeks. Fecal microbiota, fecal concentrations of organic acids and putrefactive products, fecal odor, and serum uremic toxin concentrations were evaluated before intake (0 weeks), during the 8-week intake period (4 and 8 weeks), and 4 weeks after intake (12 weeks). The activity of *N*-benzoyl-DL-arginine peptidase in dental plaque, which may be associated with periodontal disease, was evaluated at 0 and 8 weeks. Continuous intake of GOS resulted in changes in fecal microbiota, with a particularly marked increase in the abundance of *Megamonas*, which produces propionic acid. Other findings included a significant increase in the fecal acetic, propionic, and *n*-butyric acid concentrations. Additionally, significant decreases in fecal odor, fecal phenol concentration, and serum indoxyl sulfate concentration. Intake of GOS was also associated with a significant decrease in *N*-benzoyl-DL-arginine peptidase activity in dental plaques. These results suggest that continuous intake of GOS may contribute to canine health.

Key words: galacto-oligosaccharides, dog, microbiota, organic acid, putrefactive products, uremic toxins, oral environment

INTRODUCTION

Recent advances in genome analysis technology have revealed that bacterial populations in various parts of the body (i.e., the microbiome) affect digestion, immunity, and other bodily functions and also play a role in the health and diseases of their hosts [1]. Microbiome research is also attracting attention in the veterinary field, where associations between the microbiome and various diseases, including canine atopic dermatitis [2] and gastrointestinal diseases [3], have been reported. In dogs, as in humans, probiotics, prebiotics, and synbiotics are utilized to regulate the intestinal microbiome [4]. Prebiotics are food materials that are selectively metabolized by beneficial intestinal bacteria, such as bifidobacteria and lactic acid bacteria, and that regulate the intestinal microbiota by increasing or activating these beneficial bacteria, thereby contributing to maintenance of the host's health [5]. Oligosaccharides are typical prebiotics and are often added to dog food not only because of their functional properties but also because they are heat-resistant and easily processed. They reach the large intestine undigested and are metabolized by intestinal bacteria into organic acids such as acetic acid, butyric acid, and propionic acid [6]. These organic acids have been shown to contribute to the maintenance of intestinal homeostasis by inhibiting the abnormal growth of pathogenic bacteria, preventing infectious diseases, maintaining the intestinal mucosa, and regulating the immune system [7].

Although carbohydrate-derived enterobacterial metabolites have beneficial effects on host health, as described above,

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protein-derived enterobacterial metabolites such as ammonia, phenol, and hydrogen sulfide have been implicated in the etiology of intestinal diseases, especially inflammatory bowel disease (IBD) and colorectal cancer [8–10]. Phenol, indole, and *p*-cresol are also known to form sulfate conjugates in the liver and colonic mucosa; these conjugates can enter the bloodstream and damage the kidneys, thereby causing chronic kidney disease [11–13]. Thus, a wide range of evidence has indicated close relationships of the intestinal microbiota and its metabolites with diseases, suggesting that balancing the intestinal microbiota and maintaining a favorable intestinal environment are important for health maintenance and disease prevention.

Galacto-oligosaccharides (GOS) are isomerized sugars consisting of a glucose terminal and multiple galactose molecules linked together and are found in human breast milk and cow's milk [14]. In human clinical trials, GOS have been shown to be useful prebiotics that increase the number of bifidobacteria in the intestine and improve defecation frequency [15, 16]. GOS are expected to provide similar benefits in dogs and have been investigated in several canine clinical studies. However, these studies all involved dogs that were kept in research facilities and had similar backgrounds in terms of rearing conditions and diet [17–19]. Dogs within the same facility are likely to have similar microbiota compositions because the microbiota is strongly influenced by diet and can easily be transmitted among individual dogs due to their coprophagic behavior [20, 21]. Therefore, it cannot be excluded that there was bias in the initial microbiota compositions in these studies, which might have also affected the outcomes. Moreover, although there have been reports evaluating the effects of GOS intake on the canine intestinal microbiota and its metabolites, such as organic acids, separately, no study to date has comprehensively evaluated these effects [17–19].

The aim of this study was to evaluate the effects of continuous intake of GOS on the intestinal microbiota and its metabolites in healthy household dogs with diverse rearing environments. The effects of GOS on the oral environment were also evaluated.

METHODS

Animals

This study involved healthy dogs kept in households and was conducted with the cooperation of the Kawasaki Veterinary Medical Association. The study protocol was approved by the Kawasaki Veterinary Medical Association. The inclusion criteria were as follows: no antibiotic use for at least 2 weeks prior to beginning the study, no disease requiring medical treatment during the study period, no health risks associated with fecal or blood collection, age 1 year or older, owner's consent to sample collection, availability of medical history and other information deemed necessary for the study, and no use of probiotics or prebiotic supplements or discontinued use of them prior to beginning the study. Owners were instructed not to change their dogs' diets or lifestyles during the study period and not to give them supplements containing probiotics, prebiotics, or antibiotics. Written informed consent was obtained from the owners of all dogs prior to enrollment.

Study protocol

The study was conducted as an open study. A sugar syrup with at least 55% GOS, with 4'-galactosyllactose (galactoseβ1-4galactoseβ1-4glucose; Galβ1-4Galβ1-4Glc) as the main component in solids [22], was obtained from Yakult Pharmaceutical Industry Co., Ltd. and used as the test food. Parameters were compared at prescribed time points before, during, and after intake of the test food (Fig. 1). Twenty-seven dogs were fed the GOS-containing syrup in amounts according to their body weights for 8 weeks: 1.2 g (equivalent to 0.5 g GOS) per day for those weighing <5 kg, 2.4 g (1.0 g GOS) per day for those weighing ≥5 to <10 kg, and 4.8 g (2.0 g GOS) per day for those weighing ≥10 kg. Fresh feces and blood were collected before (0 weeks [w]), during (4 w and 8 w), and after (12 w) intake of the GOS-containing syrup. Feces were stored at −20°C immediately after collection and then transferred to a storage unit kept at −80°C within 3 days. Blood was immediately processed after collection to separate the serum, which was then stored at −80°C. At weeks 0 and 8, in addition to feces and blood, dental plaque samples were collected by swabbing the maxillary canine or molar teeth in the vicinity of the gingiva. Blood and dental plaque samples were collected after at least 8 hr of fasting.

Fecal processing and DNA extraction

Zirconia beads were added to fecal samples diluted 10-fold in RNAlater™ Stabilization Solution (Thermo Fisher Scientific Inc., Waltham, MA, USA) and homogenized (1,048 rpm, 10 min) using a bead homogenizer (ShakeMaster Auto BMS-A20TP, Bio Medical Science, Tokyo, Japan). Then, 200 µL of the suspension was collected, mixed with 1 mL of PBS(−), and centrifuged (13,000 G × 5 min, 4°C), and the supernatant was subsequently

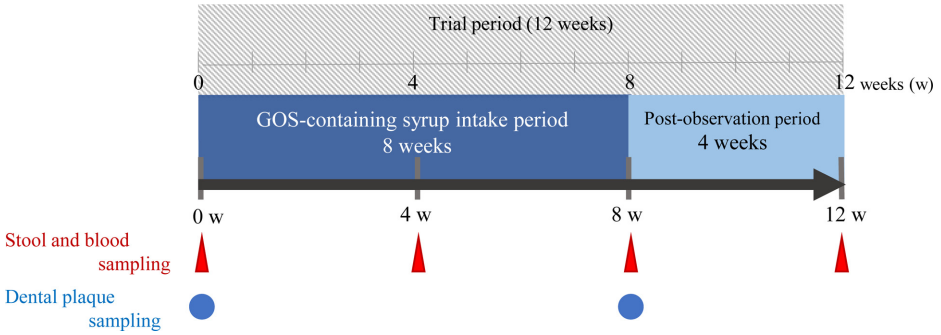

Fig. 1. Study outline.
GOS: galacto-oligosaccharides.

discarded. The same procedure was repeated, and the resulting fecal pellet was used for DNA extraction. DNA was extracted as previously described and dissolved in 0.2 mL of Tris-EDTA (TE) buffer (pH 8.0) [23]. The concentration of the obtained DNA was measured with a trace spectrophotometer (DS-11, DeNovix Inc., Wilmington, DE, USA), adjusted with TE buffer (pH 8.0) to a concentration of 5 ng/μL, and used for amplicon analysis of the 16S rRNA gene.

Amplicon analysis of the 16S rRNA gene

The composition of the fecal microbiota was determined by amplicon sequencing of the 16S rRNA gene. Specifically, 10 ng of the DNA extracted from feces was amplified using a KAPA HiFi HotStart Ready Mix PCR kit (KAPA Biosystems, Woburn, MA, USA) and purified using AMPure XP beads (Beckman Coulter, Brea, CA, USA). The following primers were used to amplify the V4 region of the 16S rRNA gene: forward (515F), 5′-GTGCCAGCMGCCGCGGGTAA-3′, and reverse (806R) 5′-GGACTACHVGGGGTWTCTAAT-3′ [24]. Library quality was assessed using an Agilent 2200 TapeStation (Agilent Technologies Inc., Santa Clara, CA, USA), and library pools were subjected to 300 cycles of single-end sequencing using a MiniSeq sequencing system and MiniSeq Mid Output Kit (Illumina, San Diego, CA, USA). The reads were imported into QIIME 2 (ver. 2022.2) for processing of the sequencing data and then assigned to taxonomic groups, using the SILVA 138 database [25]. The weighted UniFrac distance and α-diversity indices (Faith's phylogenic diversity [PD], Chao1 index, and Shannon index) were calculated using the minimum number of reads in the analyzed samples (depth, 11,863).

Measurement of fecal organic acid concentrations

The fecal concentrations of organic acids (formic acid, acetic acid, propionic acid, isobutyric acid, *n*-butyric acid, isovaleric acid, *n*-valeric acid, succinic acid, and lactic acid) were determined as described by Zhang *et al.* [26], using fecal samples pre-processed according to the method described by Asahara *et al.* [27]. A high-performance liquid chromatography (HPLC) system (LC-20AD, Shimadzu Corp., Kyoto, Japan) was used, with two Shim-pack SCR-102H columns (300 mm length × 8.0 mm inner diameter [i.d.]; Shimadzu Corp.) connected to SCR-102H guard columns (50 mm length × 6.0 mm i.d.; Shimadzu Corp.). A CDD-10Avp conductivity detector (Shimadzu Corp.) was used. The LabSolutions software (ver. 5.90; Shimadzu Corp.) was used for data analysis. The total organic acid concentration was determined as the combined concentration of the nine organic acids listed above.

Measurement of fecal putrefactive product concentrations

The fecal concentrations of putrefactive products (phenol, indole, and *p*-cresol) were determined by HPLC. Approximately 0.1 g of feces was taken and suspended in a 9-fold volume of 100 mM PIPES buffer (pH 6.7). After adding 3.6 μL of 70% perchloric acid to 250 μL of the fecal diluent, the mixture was allowed to stand overnight and then processed by salting-out-assisted liquid–liquid extraction. Then, 2 μL of the resulting sample was applied to an HPLC system (ACQUITY UPLC I-Class system, Waters Corp., Milford, MA, USA), and detection was performed using an ACQUITY UPLC PDA detector (Waters Corp.). Atlantis Premier BEH C18 AX VanGuard FIT columns (2.1 mm i.d. × 100 mm, 1.7 μm; Waters Corp.) were used, with the temperature set at 30°C. The mobile phase was composed of 10 mM ammonium acetate (AA)-acetonitrile (ACN; 90:10) as solvent A and 20 mM AA-ACN (50:50) as solvent B, and a gradient was run from 100% to 0% solvent A over 7 min at a flow rate of 0.35 mL/min. The MassLynx software (ver. 4.1; Waters Corp.) was used for data analysis.

Measurement of serum uremic toxin concentrations

The serum concentrations of uremic toxins (phenyl sulfate, indoxyl sulfate, and *p*-cresyl sulfate) were determined by HPLC mass spectrometry, using the method described by Kawase *et al.* [28]. The measurement of these substancses was outsourced to the Kyoto Institute of Nutrition & Pathology, Inc. (Kyoto, Japan)

Questionnaire survey on fecal odor

A questionnaire survey of owners was conducted to evaluate the fecal odor experienced during fecal collection on a 5-point scale, with 5 indicating "very smelly" and 1 indicating "not smelly".

Evaluating N-benzoyl-DL-arginine peptidase activity in dental plaque

N-Benzoyl-DL-arginine peptidase activity in dental plaque samples was measured with an AD*plit*® kit (Kyoritsu Seiyaku Corp., Tokyo, Japan) and evaluated on a 5-point scale, in accordance with the provided protocol.

Subgroup analysis

To evaluate the response of the dogs to the GOS-containing syrup based on their backgrounds, a subgroup analysis was performed. Age was divided into two groups based on the median value (<9.5 years, n=13; >9.5 years, n=13), and sex was divided into three groups (intact males, n=3; desexed males, n=14; expectant females, n=9). Body weight was also divided into three groups based on the initial values according to the category of GOS dosage (<5 kg, n=12; ≥5 to <10 kg, n = 9; and ≥10 kg, n=5).

Statistical analysis

All statistical analyses were performed using EZR (ver. 1.61) or R (ver. 4.2.2) software. Changes in the abundance of each bacterial genus (average abundance >0.01%) were analyzed using the ALDEx2 package [29]. Alpha diversity indices (Faith's PD, Chao1 index, and Shannon index), fecal concentrations of organic acids and putrefactive products, serum uremic toxin concentrations, fecal odor, and AD*plit*® scores were analyzed using the Wilcoxon signed-rank test. The composition of the microbiota was determined using principal coordinate analysis based on the weighted UniFrac distance and was compared using permutational multivariate analysis of variance. All of these analyses were pre- and post-intake comparisons using pre-intake data (0 w) as the baseline, with multiplicity correction using the Benjamini–Hochberg method for the weighted UniFrac distance and ALDEx2 analysis and using the Bonferroni method for the other analyses, except for the AD*plit*® scores. A p-value with multiplicity correction or we.eBH (the expected Benjamini–Hochberg corrected p-value of Welch's t-test) of <0.05 was considered to indicate statistical significance, and a p-value with multiplicity correction or we.eBH of ≥0.05 but <0.1 was considered to indicate marginal significance. For AD*plit*®

scores, a p-value of <0.05 was considered to indicate statistical significance.

In the subgroup analysis, comparisons of age and body weight among two subgroups were performed using Welch's t-test, and those among three subgroups were performed by one-way analysis of variance Sex was analyzed using Fisher's exact test. Comparisons of fecal concentrations of organic acids and putrefactive products, serum uremic toxin concentrations, fecal odor, and AD*plit*® scores among two subgroups were performed using the Mann–Whitney U test, and those among three subgroups were performed using the Kruskal–Wallis test. For all variables, a p-value of <0.05 was considered to indicate statistical significance.

RESULTS

Animals

A total of 27 dogs were enrolled in the study. During the second week of the study, one dog was withdrawn by its owner due to a disease unrelated to intake of the test food, and the remaining 26 dogs completed the study and were included in the analysis. Their background information is shown in Supplementary Table 1. The GOS-containing syrup intake rate of the 26 dogs included in the analysis was 99.0% ± 2.7%. During the study period, all dogs were found to be in good health based on the results of blood biochemistry tests and veterinary examinations, and no adverse events related to the consumption of the GOS-containing syrup, such as diarrhea, were observed.

Effects on fecal microbiota

There was a significant decrease in the Shannon index during the intake period (8 w) compared with baseline (0 w), whereas no significant changes were found in Faith's PD or the Chao1 index (Fig. 2A). Figure 2B shows the changes in the microbiota as represented by the weighted UniFrac distance from baseline (0 w) to 4 and 8 w during the intake period and then to 4 weeks after the end of intake (12 w) and indicates that the composition during the intake period (8 w) showed marginally significant differences from baseline. Figure 2C shows the changes in the abundance of the top bacterial genera. *Megamonas* showed a marked increase in abundance. To examine the effect of the GOS-containing syrup on the microbiota in more detail, a compositional differential abundance analysis of each genus was performed using ALDEx2, and the results revealed a significant increase in *Megamonas* during the intake period (4 w and 8 w) compared with baseline (0 w; Table 1). Although multiplicity-corrected results showed no significant differences, an increase in *Corynebacterium* and *Actinomyces* and decrease in *Terrisporobacter* were observed during the intake period. In addition, an increase in *Lachnoclostridium* as well as a decrease in *Lachnoclostridium*, *Escherichia-Shigella*, and *Erysipelatoclostridium* were observed 4 weeks after the end of intake (12 w).

Effects on fecal metabolites

Changes in the concentrations of the three major organic acids in feces (acetic acid, propionic acid, and *n*-butyric acid) are shown in Fig. 3A. Significant increases in the concentrations of acetic acid and *n*-butyric acid were observed at 4 w during the intake period and in the concentrations of propionic acid at 4 w and 8 w during the intake period compared with baseline (0 w). The concentrations of these organic acids were also significantly higher 4 weeks after the end of intake (12 w) compared with baseline (0 w). There were also significant increases in the concentrations of succinic acid, lactic acid, formic acid, isobutyric acid, and isovaleric acid during the intake period (Supplementary Table 2). In contrast, phenol, a putrefactive product, showed a significant decrease in concentration during the intake period (4 w and 8 w) compared with baseline (0 w) and a decreasing trend for 4 weeks after the end of intake (12 w; Fig. 3B). A decreasing trend in indole concentration was observed during the intake period (8 w), whereas no significant change in *p*-cresol concentration was observed during the study period.

Effects on serum uremic toxin concentrations

There was a significant decrease in serum indoxyl sulfate concentration during the intake period (4 w and 8 w) compared with baseline (0 w), whereas no significant changes were observed in phenyl sulfate or *p*-cresyl sulfate concentrations during the study period (Fig. 4).

Effects on fecal odor

A significant decrease in fecal odor score was observed after 8 weeks of intake (8 w, 2.27 ± 1.04; 12 w, 2.38 ± 1.10) compared with baseline (0 w, 3.12 ± 0.99; Fig. 5).

Effects on N-benzoyl-DL-arginine peptidase enzyme activity in dental plaque

There was a significant decrease in the score for *N*-benzoyl-DL-arginine peptidase activity in dental plaque samples (AD*plit*® scores) after GOS-containing syrup intake compared with baseline (0 w, 3.58 ± 1.45; 8 w, 3.12 ± 1.18; Fig. 6).

Subgroup analysis

There were no significant differences in the effects of GOS-containing syrup intake, except for the fecal phenol concentration, in relation to the background factors of age, sex, and body weight (Supplementary Table 3). The decrease in phenol concentration in the ≥5 to <10 kg subgroup was significantly greater than in the other two subgroups (<5 kg and ≥10 kg subgroups) during the intake period (4 w and 8 w) and at 4 weeks after the end of intake (12 w).

DISCUSSION

This study evaluated the effects of continuous intake of a GOS-containing syrup on the intestinal microbiota and its metabolites in healthy dogs kept in households. The results showed that continuous intake of the GOS-containing syrup for 8 weeks altered the intestinal microbiota, thereby contributing to an increase in fecal organic acid concentrations as well as a decrease in fecal putrefactive product concentrations and serum uremic toxin concentrations. Similar to the present study, several studies have investigated the functionality of GOS in dogs. However, those studies involved dogs with similar backgrounds in terms of rearing conditions and diet and thus were likely to have been conducted with a biased microbiota composition [17–19]. Furthermore, those studies did not capture changes in the microbiota or organic acids and seldom evaluated the effects of GOS on intestinal toxic metabolites, such as putrefactive products and serum uremic toxins. Therefore, the present study

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is meaningful in that it has demonstrated the efficacy of GOS, in terms of the changes in these parameters, in healthy household dogs with diverse rearing environments. This study also found a decrease in *N*-benzoyl-DL-arginine peptidase activity, which has been suggested to be associated with periodontal disease, in dental plaque samples during the GOS-containing syrup intake

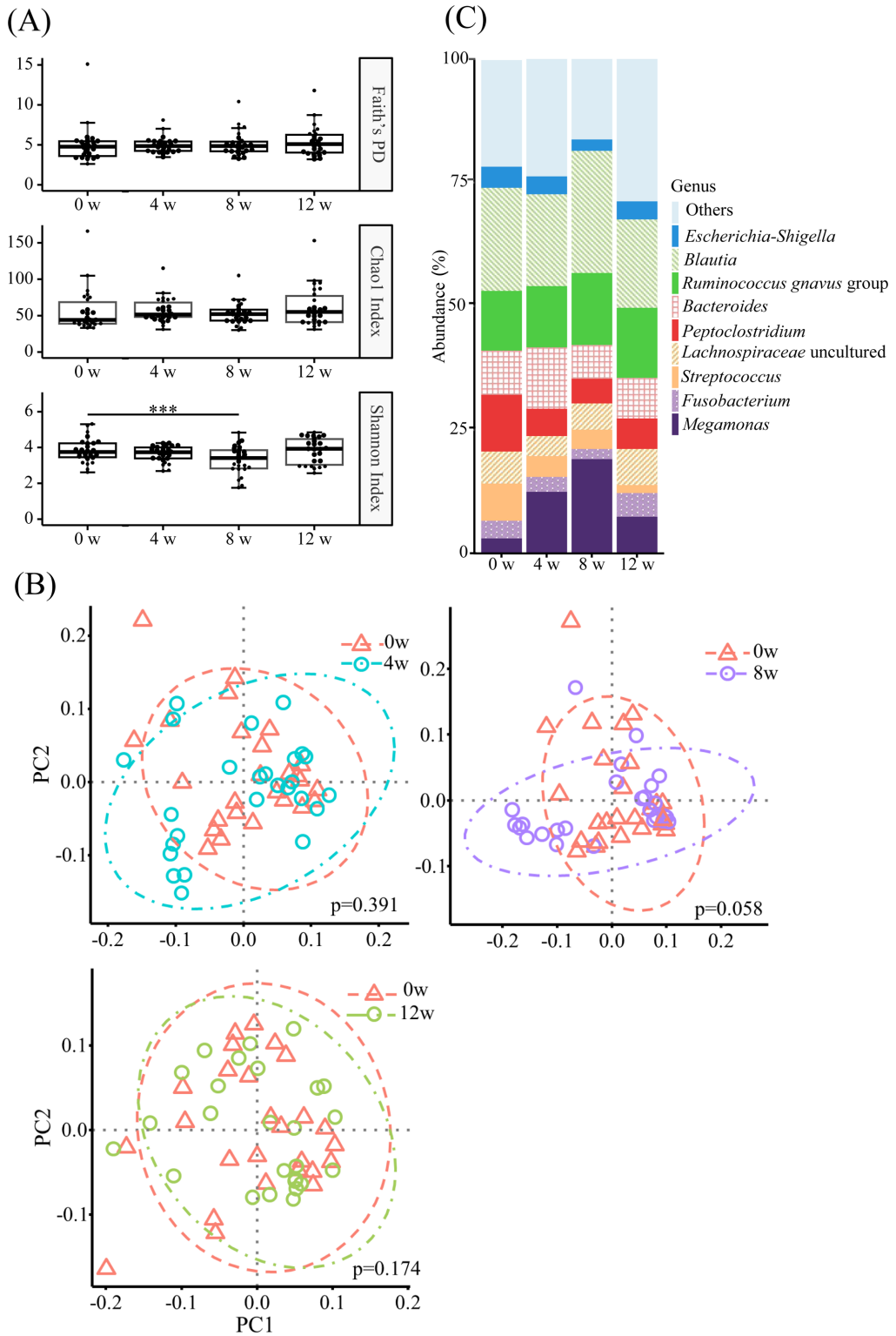

Fig. 2. Changes in diversity of the fecal microbiota during the study period. (A) Alpha diversity. (B) Beta diversity. (C) Intestinal abundance. Alpha diversity data are expressed as box plots, Beta-diversity data are expressed as PCoA plots based on the weighted UniFrac distance, and abundance data are expressed as means. Dashed lines indicate 95% confidence intervals. n=26. Significant differences in Alpha diversity and Beta diversity were determined using the Wilcoxon signed-rank test with Bonferroni correction and PERMANOVA with Benjamini–Hochberg correction, respectively (vs. 0 w). ***p<0.001. PD: phylogenic diversity.

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Table 1. Centered log-ratio transformation values of the occupancy of intestinal microbiota (Clr) that showed significant changes before and after galacto-oligosaccharides (GOS)-containing syrup intake

Genus	Clr (median [Q1,Q3])				we.ep[1] value for 0 w			we.eBH[2] value for 0 w		
	0 w	4 w	8 w	12 w	4 w	8 w	12 w	4 w	8 w	12 w
Megamonas	−1.53 [−1.74,5.15]	3.15 [1.83,7.69]	4.31 [3.10,8.86]	2.89 [−1.72,6.14]	<0.001	<0.001	0.098	0.005	0.001	0.382
Corynebacterium	−1.76 [−2.33,−1.58]	1.98 [−1.87,2.70]	−1.68 [−1.99,1.82]	−0.05 [−1.72,1.66]	0.012	0.273	0.059	0.123	0.626	0.264
Actinomyces	−1.72 [−2.19,−1.50]	1.15 [−2.00,1.68]	−1.25 [−2.01,1.28]	−1.68 [−2.20,1.09]	0.031	0.202	0.482	0.201	0.554	0.729
Lachnoclostridium	5.05 [3.52,6.62]	5.49 [4.61,6.58]	6.01 [4.42,6.70]	6.51 [4.90,7.17]	0.057	0.091	0.018	0.331	0.484	0.192
Terrisporobacter	2.93 [−1.73,5.71]	0.22 [−2.18,3.82]	−1.70 [−1.95,2.12]	0.10 [−2.33,3.61]	0.204	0.039	0.086	0.524	0.285	0.365
Escherichia-Shigella	6.00 [3.18,6.84]	5.33 [−0.85,6.67]	5.29 [−0.24,6.52]	4.49 [−2.21,6.32]	0.125	0.098	0.016	0.448	0.461	0.193
Erysipelatoclostridium	3.43 [2.62,4.22]	2.70 [1.69,4.42]	3.06 [2.30,4.27]	4.04 [3.30,5.24]	0.678	0.464	0.019	0.864	0.464	0.206

[1]Welch's t-test p-value.
[2]Benjamini–Hochberg corrected p-values for we.ep.

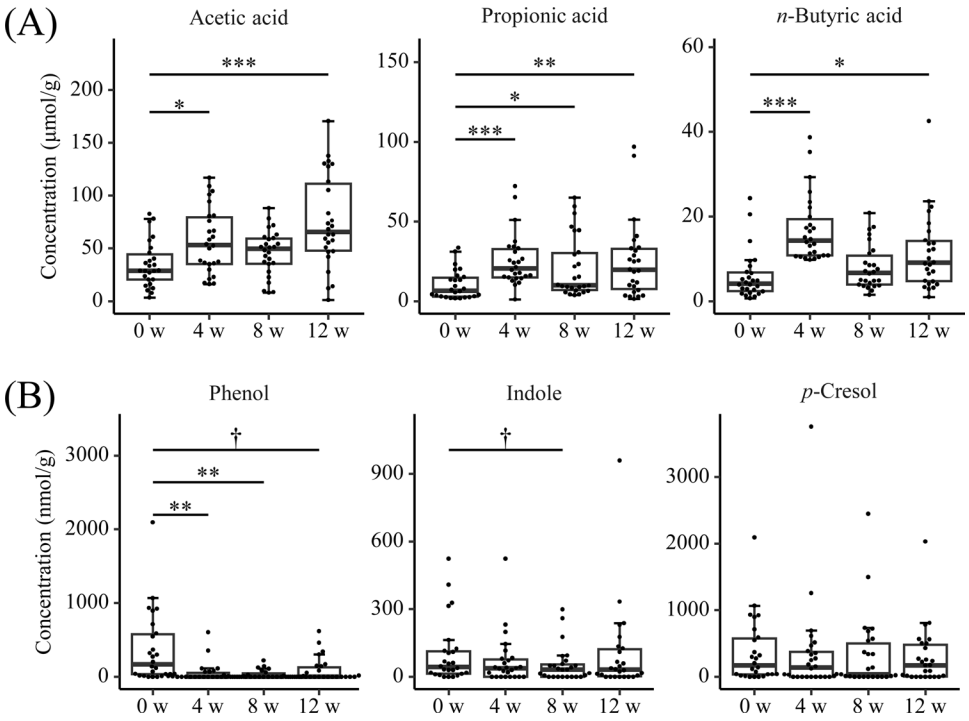

Fig. 3. Changes in the concentrations of fecal organic acids and putrefaction products during the study period. (A) Organic acid concentrations. (B) Putrefaction product concentrations. Data are expressed as box plots. n=26. Significant differences were determined using the Wilcoxon signed-rank test with Bonferroni correction (vs. 0 w). †p<0.10; *p<0.05; **p<0.01; ***p<0.001.

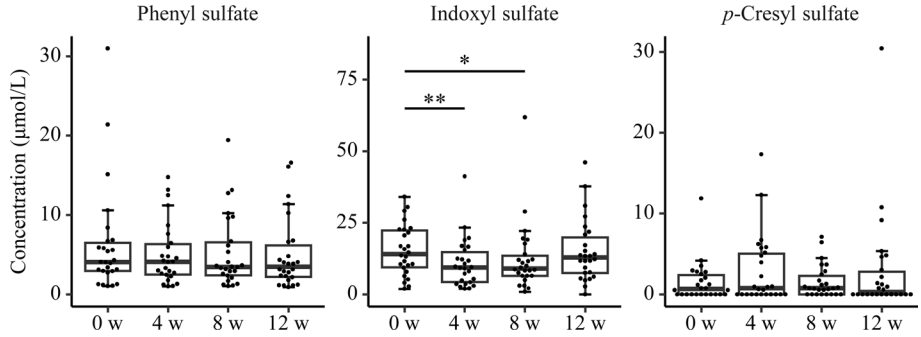

Fig. 4. Changes in serum levels of uremic toxins during the study period. Data are expressed as box plots. n=26. Significant differences were determined using the Wilcoxon signed-rank test with Bonferroni correction (vs. 0 w). *p<0.05; **p<0.01.

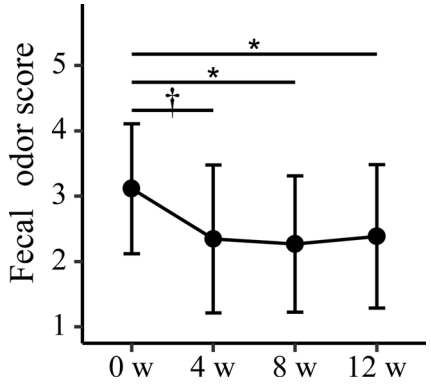

Fig. 5. Changes in fecal odor score during the study period. Data are expressed as means ± SD. n=26. Significant differences were determined using the Wilcoxon signed-rank test with Bonferroni correction (vs. 0 w). †p<0.10; *p<0.05. SD: standard deviation.

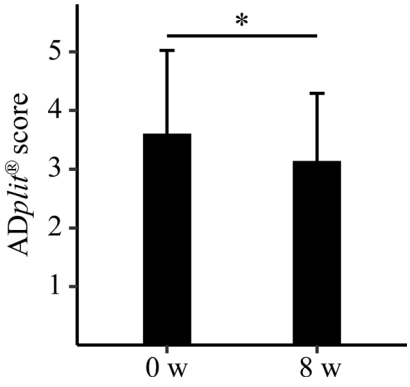

Fig. 6. Changes in AD*plit*® score during the trial period. Data are expressed as means ± SD. n=26. Significant differences were determined using the Wilcoxon signed-rank test (vs. 0 w). *p<0.05. SD: standard deviation.

period. To our knowledge, this effect of continuous intake of GOS has not previously been reported and is thus a new finding.

Continuous intake of the GOS-containing syrup decreased the alpha diversity (Shannon index) of the fecal microbiota during the intake period, affecting the composition of the microbiota (Fig. 2A). An increase or decrease in abundance was also observed in multiple genera of bacteria (Table 1). In particular, *Megamonas* was most affected by GOS-containing syrup intake, showing a marked increase in abundance (Fig. 2C). *Megamonas* are the major propionic acid-producing bacteria in the intestine and have been reported to exhibit β-galactosidase activity [30, 31]. The significant increase in *Megamonas* abundance and fecal propionic acid concentration observed in this study suggest that *Megamonas* bacteria proliferated and produced propionic acid through the utilization of GOS. Previous studies have reported that, compared with the abundance in healthy dogs, the abundance of *Megamonas* is decreased in dogs with atopic dermatitis or myxomatous mitral regurgitation [2, 32]. Continuous intake of GOS may contribute to the prevention of these diseases and the improvement of their symptoms. In atopic dermatitis, immune imbalance has been implicated in the onset or worsening of the disease. The present study showed not only an increase in the production of propionic acid, which has an anti-inflammatory effect, via the proliferation of *Megamonas* but also a significant increase in *n*-butyric acid, which is involved in the induction of regulatory T cell differentiation, suggesting the potential efficacy of GOS against atopic dermatitis (Fig. 3A) [7]. In addition to acetic acid, propionic acid, and *n*-butyric acid mentioned above, several other organic acids were also significantly increased during the GOS-containing syrup intake period (Supplementary Table 2). These results suggest that GOS can be utilized by multiple species of bacteria with different organic acid-producing systems in the intestine. Furthermore, some organic acids remained at high levels for 4 weeks after the end of intake (12 w) compared with baseline (0 w). For propionic acid, the involvement of *Megamonas*, a propionic acid-producing bacteria that was highly, albeit not significantly, abundant even at 12 weeks, is considered noteworthy. In contrast, no significant changes were observed in the bacteria responsible for the production of the other organic acids, suggesting that multiple bacteria may be involved in a complex manner. Further studies are needed to elucidate these mechanisms.

As mentioned earlier, a decrease in fecal alpha diversity was observed during the GOS-containing syrup intake period. This finding may be attributable to a decrease in intestinal pH due to the increase in organic acids, resulting in a decrease in the number of susceptible bacterial species. This study also showed a significant decrease in the fecal phenol and indole concentrations and the serum indoxyl sulfate concentration with GOS-containing syrup intake (Figs. 3B and 4). These are intestinal bacterial metabolites derived from tyrosine and tryptophan in proteins [33]. Phenol, which has been implicated in colorectal cancer, has been reported to increase colorectal epithelial cell permeability in a concentration-dependent manner, while a positive correlation has been observed between DNA damage in colon tissue and the fecal phenol concentration [10, 33]. Indole is reported to be a causative agent of fecal odor [34]. In the present study, GOS-containing syrup intake also improved fecal odor, presumably due to a decrease in indole concentration (Fig. 5). Indole molecules absorbed from the intestinal tract are undergo sulfate conjugation in the liver and are released into the blood as indoxyl sulfate. Indoxyl sulfate has been shown to be associated with chronic kidney disease, and a decreased concentration of this substance through continuous intake of GOS is expected to contribute to the prevention of chronic kidney disease and to suppress the decline in renal function [13, 35]. Meanwhile, the present study did not clarify the relationships of the decreases in these putrefactive products and uremic toxins with the changes in the microbiota. Given the increases in various organic acids, this finding may be attributable to changes across multiple bacterial species rather than in a specific species, although the mechanism remains unclear. Further analyses, such as RNA-seq, are needed to clarify the underlying mechanisms.

N-Benzoyl-DL-arginine peptidase is an enzyme specifically produced by *Porphyromonas glae*, which is frequently detected in periodontal lesions in dogs, as well as *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*, which are grouped as red-complex bacteria in human periodontal diseases. The activity of *N*-benzoyl-DL-arginine peptidase has been correlated not only with the number of the abovementioned

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bacteria but also with the severity parameters of periodontal disease, including the number and depth of periodontal pockets, as well as with halitosis [36–38]. In the present study, continuous intake of the GOS-containing syrup resulted in a decrease in the activity of the enzyme in dental plaque samples (Fig. 6). These results indicate the potential contribution of GOS to the prevention and improvement of periodontal disease and oral malodor. However, the mechanism underlying this effect remains unclear, warranting further investigation.

This study involved healthy household dogs with diverse rearing environments to avoid the situation in which dogs kept in the same facility potentially have similar microbiota. As a result, GOS intake was shown to provide benefits to a wide-ranging population with diverse rearing environments. In addition, the subgroup analysis based on the background factors of age, sex, and body weight showed that the effects of GOS were not influenced by those factors, except for the effect of body weight on the fecal phenol concentration (Supplementary Table 3). However, the baseline level of the fecal phenol concentration in the ≥5 to <10 kg subgroup, in which the reduction effect was particularly strong, was notably higher than those in the other subgroups (Supplementary Fig. 1). However, the other subgroups also showed a reduction, implying that the effect was not attributable to body weight. These findings indicate that GOS intake in dogs is beneficial regardless of the rearing environment and background factors.

This study has several limitations. First, it was a single-arm open study, and thus a double-blind, parallel-group study with a placebo would be needed to obtain data with a higher level of evidence regarding the effectiveness of the GOS-containing syrup. Second, the GOS-containing syrup used in this study was a sugar syrup produced industrially using enzymatic reactions, and 45% of its solid component consisted of the monosaccharides Glc and Gal, as well as the disaccharide Galβ1-4Glc [22]. Monosaccharides are digested and absorbed in the upper gastrointestinal tract, suggesting that they have little impact on the microbiota. The disaccharide Galβ1-4Glc also has little influence [39]. Consequently, the effects observed in this study are assumed to be attributable to GOS. However, evaluation using a test food with a higher GOS purity would be useful in clarifying the effects of GOS.

In conclusion, this study demonstrated that continuous intake of a GOS-containing syrup for 8 weeks in healthy dogs kept in households with diverse rearing environments in terms of diet and other factors altered the intestinal microbiota, improved the intestinal environment, and reduced oral *N*-benzoyl-DL-arginine peptidase activity. These results suggest that continuous intake of GOS may contribute to health maintenance and disease prevention in dogs.

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