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FULL PAPER

Toxicology

Surveys of eleven species of wild and zoo birds and feeding experiments in white-tailed eagles reveal differences in the composition of the avian gut microbiome based on dietary habits between and within species

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ABSTRACT. The composition of the gut microbiome varies due to dietary habits. We investigated influences of diet on the composition of the gut microbiome using the feces of 11 avian species, which consumed grain-, fish- and meat-based diets. We analyzed gut microbiome diversity and composition by next-generation sequencing (NGS) of 16S ribosomal RNA. The grain-diet group had higher gut microbiome diversity than the meat- and fish-diet group. The ratio of *Bacteroidetes* and *Firmicutes* phyla was higher in the grain-diet group than in the meat- and fish-diet groups. The grain-diet group had a higher ratio of *Veillonellaceae* than the meat-diet group and a higher ratio of *Eubacteriaceae* than the fish-diet habit group. To clarify the influence of diet within the same species, white-tailed eagles (*Haliaeetus albicilla*, n=6) were divided into two groups, and given only deer meat or fish for approximately one month. The composition of the gut microbiome of individuals in both groups were analyzed by NGS. There were indications of fluctuation in the levels of some bacteria (*Lactobacillus*, *Coriobacteriales*, etc.) in each diet group. Moreover, one individual for each group which switched each diet in last week changed to each feature of composition of bacterial flora. The above results show that the composition of the gut microbiome differ depending on diet, even within the same species.

KEYWORDS: avian, dietary habitat, microbiome, next-generation sequencing, white-tailed eagle

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The gut microbiome refers to the complex ecosystem comprising the bacteria living in the intestinal tract. Development of gut microbiome mostly depends on maternal (mammals) or egg shell and nest environmental (oviparous animals) transmission [17]. The gut microbiome interacts with the host and has many ways of affecting the host's body, with the degree of influence on the host depending on the composition of the gut microbiome. Digestion, the immune system, and disease risk are well-known examples of systems that are influenced by the gut microbiome [1].

The presence, absence, and fluctuation of each bacterium in the microbiome depends on various factors. These factors are broadly classified as either external or internal: external factors include habitats, feed resources, behaviors, lifestyles, soils, and seasons; whereas internal factors refer to diets, host strains, digestive tract morphology, sex, and age [12]. These factors appear independent, but in fact influence complex elements that are relevant to the life of the host. Accordingly, in this study, we have speculated that diet exerts the greatest influence on the composition of the microbiome, because many factors that affect the microbiome are thought to include a dietary element. For example, artificially cultivated western capercaillie (*Tetrao urogallus*) have a high mortality rate after they are released into the wild because of weight loss [23]. Wild western capercaillie typically eat conifer leaves, which contain high concentrations of the toxic resin, and thus show resin resistance. However, the total length of the cecum, which is important for metabolism, is decreased in cultivated western capercaillie, compared to their wild counterparts. Furthermore, captive individuals do not have *Synergistes*, which is a beneficial bacterium that contributes to resin detoxification. Thus, cultivated western capercaillie might not be able to detoxify resin from the coniferous leaves [23].

Differences in diet, as described above, occur not only between species, which have distinct dietary tendencies, but also occur within the same species. For example, it is known that the differences in intestinal bacterial flora are observed in humans from different regions of the world are attributable to differences in diet [19]. However, if we verified the influence of diet on the gut microbiome only within a homogeneous species, we would not be able to eliminate the influence of host strain factors. Therefore, it is important to investigate both inter- and intra-species differences in dietary factors. We focused on birds in the current study, based on the assumption that it is easy to compare between species and to investigate the influences of diet for the following reasons. Various bird species consume several different food types, such as grains, meats, fish, honey, and insects [12]. In addition, with regards to the intestinal tract mechanism, birds are more likely to rely on their intestinal wall barrier than mammals. In general, mammals actively (i.e., selectively) absorb almost all nutritional components via intestinal epithelial cells. On the other hand, birds absorb glucose and amino acids actively, but absorb other components passively (i.e., non-selectively; [16]). As a result, there is a high possibility that harmful components, such as xenobiotics, are also absorbed in birds. Therefore, it has been proposed that the gut microbiome and intestinal wall barrier have important effects on digestion and metabolism in birds. Moreover, many bird species have broad range of habitat based on migration. It could be assumed that some avian gut bacteria are affected by environment. However, most research on avian gut microbiome is related to poultry such as chickens and ducks. So, it is important to understand the characteristics of the composition of various avian gut microbiome.

Given the background presented above, we examined the influences of diet on the gut microbiome composition in two experiments. The objective of experiment 1 was to categorize various avian species based on their diet, and investigate the influence of diet on gut microbiome composition independent of the host species. In this experiment, the proportions of intestinal microorganisms that differed based on the consumption of grain-, meat-, or fish-based diets were evaluated, and their relationship with food intake was examined. We predicted that the different diets would produce effects on the composition of bacterial flora. However, because many bird species were used in experiment 1, we could not eliminate the possible impact of environmental factors on the gut microbiome. Therefore, with reference to the data from experiment 1, the objective of experiment 2 was to clarify the influence of differences in diet within the same species. White-tailed eagles (WTEs), which eat both meat and fish, were used in experiment 2. The WTEs were divided into two groups and given only deer meat or fish for approximately one month. Then, we examined whether there were differences in the composition of their bacterial flora. Through experiments 1 and 2, we gained deeper insights by focusing on the factor of diet from different perspective.

MATERIALS AND METHODS

Animals and fecal sample collection

Experiment 1: Comparison between bird species: Feces were collected from Indian peafowls (*Pavo cristatus*), domestic geese (*Anser cygnoides*), ducks (*Anas platyrhynchos domesticus*), ostriches (*Struthio camelus*), night herons (*Nycticorax nycticorax*), Humboldt penguins (*Spheniscus humboldti*), and great horned owls (*Bubo virginianus*) kept at the Maruyama Zoo in Hokkaido, Japan (Table 1). Feces were collected using autoclaved spatulas and enclosed in 15 mL or 30 mL sample tubes.

Feces were collected from three WTEs and one falcon (*Falco peregrinus*) housed at the Kushiro Shitsugen Wildlife Center, Japan, which were fed frozen meat (Table 1). The falcon and WTEs feces were collected using autoclaved disposable chopsticks and placed in 30 mL sample tubes. Fecal samples collected from WTEs on their final feeding day were used for this experiment.

Feces were collected from black-tailed gulls (*Larus crassirostris*) and slaty-backed gulls (*Larus schistisagus*) on Rishiri Island, Japan (45°18' N, 141°24' E) (Table 1). The high-fluidity feces were collected using a pipette with a 1 mL pipette tip and placed in a 30 mL sample tube. The edge of the tip was cut to facilitate collection of small fragments in the fecal sample.

The ground of the animal house was basically concrete and was carefully cleaned before the study began. After feces samples were collected, they were cleaned in the same manner so that fresh feces could always be collected. For outdoor feces, fresh feces were selected as much as possible and collected so as not to be contaminated by environmental soil. All sample tubes were stored in liquid nitrogen for transfer to the laboratory. Upon arrival, samples were stored in a −80°C freezer until bacterial genomes were extracted.

Table 1. Details of samples used in experiment 1

Diet habitat	Species	Number of samples	Sample site	Diet contents
Grain	Indian peafowl (*Pavo cristatus*)	3	Maruyama Zoo	Pheasant pellt, clover, seed mix, oyster shell
	Domestic goose (*Anser cygnoides*)	2	Maruyama Zoo	Duck pellet, clover, oyster shell, vitamin a compounds
	Duck (*Anas platyrhynchos domesticus*)	1	Maruyama Zoo	Duck pellet, clover
	Ostrich (*Struthio camelus*)	2	Maruyama Zoo	Cabbage, been sprouts, ostrich pellet, oyster shell, bread, iucerne pellet
Fish	Night heron (*Nycticorax nyvticorax*)	1	Maruyama Zoo	Sandfish
	Humboldt penguins (*Spheniscus humboldti*)	5	Maruyama Zoo	Sand lance
	Slaty-backed Gull (*Larus schistisagus*)	1	Rishiri Island	Unknown
	Black-tailed Gull (*Larus crassirostris*)	3	Rishiri Island	Unknown
Meat	Great Horned Owl (*Bubo virginianus*)	1	Maruyama Zoo	Chick
	Peregrine Falcon (*Falco peregrinus*)	1	WLC	Quail chick
	White-tailed Eagle (*Haliaeetus albicilla*)	3	WLC	Deer meat

The typical feeds for individual birds are described. The wild individuals on Rishiri Island were assigned to the fish-diet group because they were force-fed fish to promote excretion, but the contents of their typical diet is unknown. WLC, Kushiro Shitsugen Wildlife Center.

Experiment 2: WTEs feeding experiment: Animal experiments were performed at the Kushiro Shitsugen Wildlife Center under supervision and with the endorsement of the Institutional Animal Care and Use Committee of Hokkaido University, Japan. Individual WTEs were housed separately in outdoor individual breeding huts, and were fed a specific diet for approximately one month (Fig. 1). Control samples were obtained from WTEs (n=7) before dietary treatment, and WTEs were fed a diet of both meat and fish. Excluding one individual, WTEs (n=6) were assigned into either the meat feeding group (meat-diet group, n=3) or the fish feeding group (fish-diet group, n=3), and fed thawed meat or fish (approximately 10 types). Feeding and fecal collection took place at around 9 a.m. daily, for about a month. The reason for month-long duration of the experiment was to verify any compositional fluctuations in the gut microbiome. According to David *et al*. [7], the diversity of bacterial flora in humans transitioning from an ordinary diet to a plant- or animal-based diet changed in only two days. However, as no published research has examined such compositional changes in birds over time, it was difficult to predict how long it would take for the gut microbiome to adapt to diets limited to either meat or fish. Therefore, the WTEs were treated with a limited diet for about one month in this experiment.

In addition, the WTEs were not force-fed, so the amount of food that each eagle consumed varied daily. On some days, the eagles did not spontaneously ingest the food, while on others, they were not fed to ensure their health. In addition, we observed a fasting period, which lasted several days after the experiment commenced, in each individual. To identify the influence more clearly, we swapped the diet of two individuals (IDs: meat2, fish2) on the 33rd and 34th days after commencing the experiment (i.e., meat2 was given fish, and fish2 was given meat). The ground of the animal house was basically concrete and was carefully cleaned before the study began. After feces samples were collected, they were cleaned in the same manner so that fresh feces could always be collected. Feces were collected daily, using autoclaved disposable chopsticks, placed in 30 mL sample tubes, and stored in liquid nitrogen until they were transferred to the laboratory. After transfer to the laboratory, samples were kept in a −80°C freezer.

Although feces were collected daily, the number of fecal samples with sufficient quality for sequencing was limited, and only 119 out of the 185 samples collected were used for the bacterial flora analysis. Furthermore, because sampling dates were inconsistent across individuals, the data were sorted into five-day averages for comparison. Term 0 includes the days until meat-only or fish-only treatment, and one of the Term 0 samples was used as the control group for each individual. Term 7 consists of the 33rd and 34th days after swapping the diets of individuals fish2 and meat2.

Next-generation sequencing

Next-generation sequencing (NGS) was performed as described below. Briefly, a QIAamp FAST DNA Stool Mini Kit (QIAGEN, Nordrhein-Westfalen, Germany) was used to extract bacterial DNA from 150–200 mg fecal samples, according to the manufacturer's instruction. A Qubit ™ 4 Fluorometer (Invitrogen™, Carlsbad, CA, USA) was used to measure the DNA concentration. PrimeSTAR® Max DNA Polymerase (TaKaRa Inc., Kusatsu, Japan) and following primers (final concentration 3 μM) were used to amplify the V4 region of 16S ribosomal RNA (rRNA): F515, 5′-GTGCCAGCMGCCGCGGTAA-3′ and R806, 5′-GGACTACVSGGGTATCTAAT-3′ [5]. PCR was performed using a thermal cycler (Life ECO, BIOER, Zhejiang, China), with the following settings: 98°C for 10 sec, 54°C for 5 sec, and 72°C for 5 sec, repeated for 30 cycles. We confirmed that the band of the first PCR product was observed at ~350 bp. Purification of PCR products was performed using AMPure XP (BECKMAN COULTER, Brea, CA, USA). A second PCR was also performed with PrimeSTAR® Max DNA Polymerase and barcode primers (forward primer, IonA-barcode[i]-F515; reverse primer, ionP1-F806; final concentration 3 μM) on the thermal cycler, with the following settings 98°C for 10 sec, 54°C for 5 sec, and 72°C for 5 sec, repeated for five cycles. We confirmed that the products of the second PCR contained a ~400 bp band. Purification and concentration measurements were performed for the products of the second PCR, using the same procedure described for the first PCR. In order to adjust the concentration of each sample more accurately, the concentration of several samples was measured using the BioAnalyzer Agilent 2100 (Agilent Technologies, Santa Clara, CA, USA), with LabChip from the DNA 1000 kit, according to the

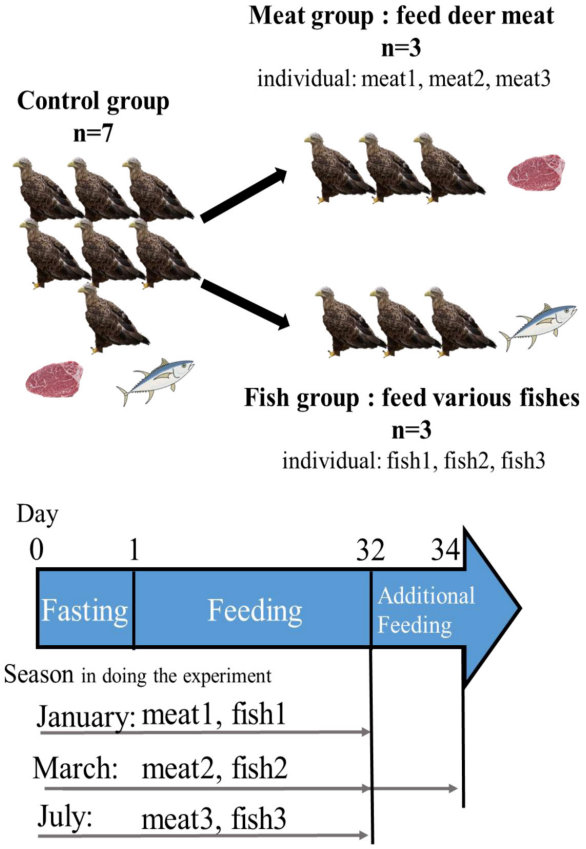

Fig. 1. Schematic of experiment 2. There were seven birds in the control group. In total, six birds underwent the diet treatments, and one bird did not. The control group was given a diet consisting of both meat and fish. The birds were fasted for one day, and food was given from the second day onwards. The number of days before each individual began the diet treatment differed. On the 33rd and 34th days after the experiment commenced, the diets of two individuals (ID: meat2, fish2) were swapped.

protocol from the manufacturer's website (https://www.chem-agilent.com/pdf/BioA_SII_DNA_v04_05_20160830.pdf).

Library preparation was performed using an Ion Chef™ Instrument and Ion PGM™ Hi-Q™ View Chef 400 Kit (Thermo Fisher Scientific, Waltham, MA, USA). To compare sample concentrations against those that were measured in Qubit, a calibration curve was generated using samples whose concentrations were measured using the Bioanalyzer. All Qubit-measured sample concentrations were corrected using the calibration curve. Equal molar amounts from each sample were used to prepare the sample mix, which was diluted to 50 pmol/L.

An Ion PGM™ System (Thermo Fisher Scientific Inc.) was used for DNA sequencing. The output data were sent to Torrent Browser (supplied by Thermo Fisher Scientific Inc.), verified, and downloaded as compressed files, which were primarily used for analysis with Qiime (1.9.1, built in Bio-Linux-8.0.7 [6]).

Data analysis was performed primarily using Qiime, R (386 3.4.1 using RStudio), and JMP (Pro 14, SAS Institute Inc., Cary, NC, USA). For Qiime, terminal commands were executed to create an operational taxonomic unit (OTU) table, and to perform α-rarefaction and β-diversity analyses (weighted and unweighted UniFrac analyses). Details of the analysis procedures for experiment 1 and 2 are described below.

Experiment 1: Comparison between bird species: Steel-Dwass tests were conducted in R, using the NSM3 package, to identify significant differences in the gut microbiome ratios among the groups of each diet habit category. A *P*-value below 0.05 was considered to indicate a significant difference. Principal coordinate analysis (PCoA) was also performed on the data from the β-diversity analysis using R.

The Shannon diversity index was calculated from the OTU table, using the "MASS" and "vegan" packages in R. The samples were categorized by diet, and Steel-Dwass tests were performed to detect differences.

Experiment 2: WTE feeding experiments: In order to investigate fluctuations in the gut microbiome, samples were categorized by each individual, normalized using the "DESeq 2" package, and compared using the OTU count number in R. When a significant difference was observed for the OTU, the composition ratio for each individual was calculated for each term, to confirm fluctuations. Steel-Dwass tests were also conducted to identify significant differences in the ratio of gut microbiome composition between the control, the meat-diet group, and the fish-diet group in R, using the "NSM3" package; $P<0.05$ was considered significant. Graphs of the gut microbiome composition for each individual were created in JMP.

To identify changes before and after completion of the diet treatment, we first used data from all seven individuals before treatment as the control, including one individual that was not assigned to either the meat-diet group or the fish-diet group. Next, the samples that were collected during the final phase of the feeding period that had the highest OTU count in each individual were identified. The average of these samples from the two diet groups was calculated. Term 7 samples were excluded. Composition graphs were created at the phylum, class, order, and family levels in JMP.

RESULTS

The results from experiment 1 showed that the composition of the bacterial flora was divided into two groups: the grain-diet group, and the meat- or fish-diet groups. Furthermore, the slight structural differences between the meat-diet group and the fish-diet group were more clearly demonstrated in a focused experiment.

Comparison between avian species (Experiment 1)

A) Diversity: α-Rarefaction analysis: Figure 2 shows the α-rarefaction analysis after grouping by diet. The blue, green, and brown lines reflect the fish, grain, and meat-diet groups, respectively. The fish- and meat-diet groups had similar trend of represents of OTUs. Whereas, the grain-diet group showed greater represents of OTUs.

Shannon index: Because rarefaction analysis showed that the gut microbiome in herbivorous birds was most diverse, we calculated the Shannon index using the OTU table (Fig. 3) and found a significant difference ($P=0.0017$) between the grain-diet and the

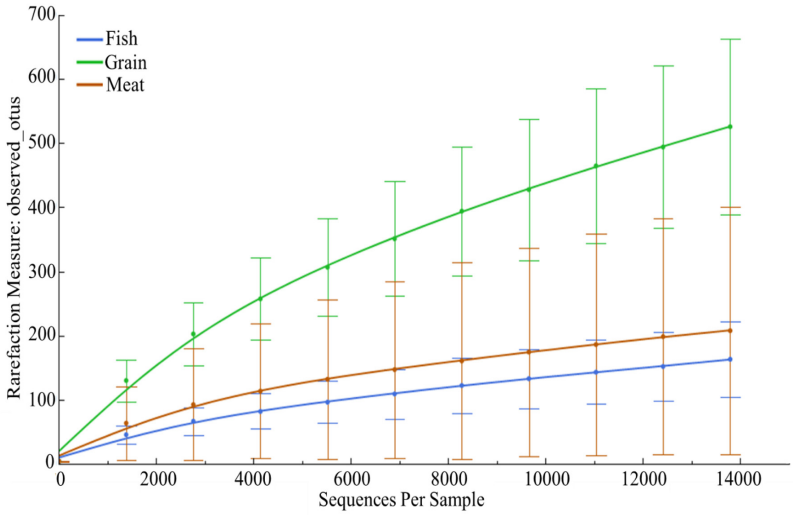

Fig. 2. The α-rarefaction analysis comparing the gut microbiome in the three diets. The vertical axis represents the number of operational taxonomic units (OTUs) observed by rarefaction analysis at each sequence number. The traces indicate the average of the analysis results for each group. Vertical lines indicate standard deviations. The fish-, grain-, and the meat-diet groups are represented by the blue, green, and brown traces, respectively.

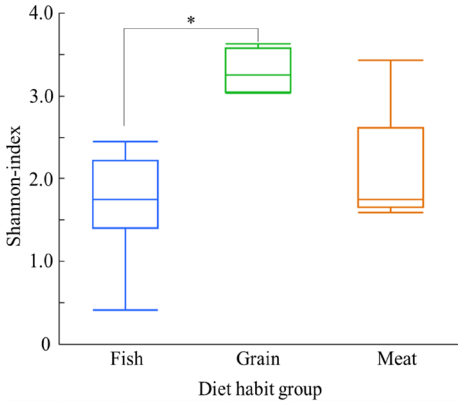

Fig. 3. Shannon diversity analysis of the three diet groups. The Shannon index was calculated and averaged for each diet group. A significant difference was observed between the fish-diet and the grain-diet groups (*Steel-Dwass test, $P<0.05$).

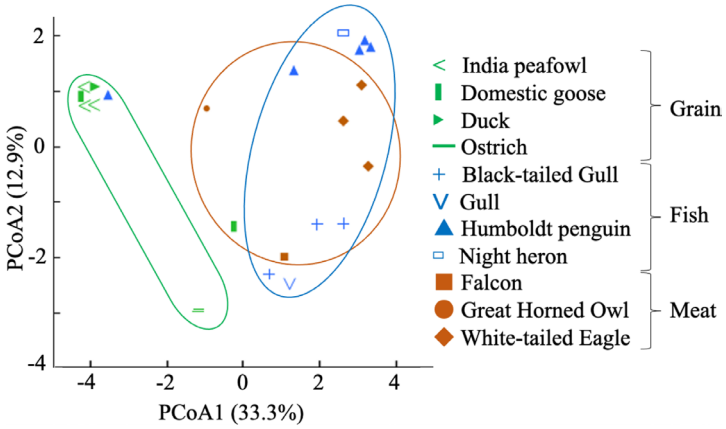

Fig. 4. Unweighted UniFrac analysis of gut microbiome composition. Different colors indicate the different feeding characteristics, with the blue, green, and brown markers representing the fish, grain, and the meat-diet habit groups, respectively. The areas that encompass many samples of each diet group are denoted by circles of the same color. The distances between samples are determined by similarity.

fish-diet groups. However, there was no significant difference between the grain-diet and the meat-diet group (P=0.0853).

Unweighted UniFrac analysis: An unweighted UniFrac analysis was conducted and plotted using PCoA, as shown in Fig. 4. The blue, green, and brown points denote the fish-, grain-, and the meat-diet groups, respectively. The PCoA 1 values differed between the grain-diet group and the fish- and meat-diet groups; the grain-diet group had negative PCoA 1 values, whereas the fish- and meat-diet groups showed near-positive values. No differences between PCoA 2 values were observed between groups, although the plot position of each sample showed rough trends based on diet. Furthermore, individuals of the same species were plotted at fairly homologous positions, depending on the host species and diet contents.

B) Gut microbiome composition: Steel-Dwass tests were conducted at each biological classification level, and the bacteria species that showed significant differences were extracted (Table 2). Many bacteria within the *Bacteroidetes*, *Tenericutes*, *Proteobacteria*, and *Firmicutes* phyla were significantly different between the grain-diet group and the fish- and meat-diet groups, and comprised a higher ratio in the OTU of the grain-diet group.

On the other hand, there was also a significant difference in some bacteria between the fish-diet and meat-diet groups. *Veillonellaceae* (phylum: *Firmicutes*) was significantly higher in the meat-diet than the grain-diet group (P=0.0152). In the *Clostridiaceae* family (P=0.0207), the proportion of *Eubacteriaceae* (P=0.0113) and *Lacnospiraceae* (P=0.0029) was significantly higher in the meat-diet group than in the fish-diet group.

Furthermore, *Flavobacteriia* were significantly higher in the fish-diet group than the grain-diet group (P=0.012).

WTEs feeding experiment (Experiment 2)

WTEs, which eat both meat and fish, were used in experiment 2. The WTEs were divided into two groups and given only deer meat or fish for approximately one month. Then, we examined whether there were differences in the composition of their bacterial flora.

Compositional ratios of bacterial flora per term in each WTEs: The variation in composition of intestinal bacterial flora for each

Table 2. Bacterial species that showed significant differences

Phylum	Class	Order	Family	*P*-value					
				Grain-Fish		Fish-Meat		Grain-Meat	
Actinobacteria	*Actinobacteria*			0.0062	G				
		Actinomycetales		0.0171	G				
			Micrococcuceae	0.0191	G				
Bacteroidetes	*Bacteroidia*			0.0183	G			0.0290	G
		Bacteroidales		<0.0001	G			0.0226	G
			Bacteroidaceae	<0.0001	G				
			Prevotellacrae					0.0306	G
			Porphyromonadaceae	0.0183	G			0.0148	G
	Flavobacteriia			0.0120	F				
		Flavobacteriales		0.0118	F				
			Flavobacteriaceae	0.0123	F				
Euryarchaeota	*Methanobacteria*							0.0122	G
		Methanobacteriales		0.0012	G			0.0127	G
			Methanobacteriaceae					0.0084	G
Firmicutes	*Bacilli*	*Bacillales*		0.0183	G			0.0083	G
			Bacillaceae					0.0085	G
		Lactobacillales	*Lactobacillaceae*	*0.0507	G			0.0261	G
	Clostridia	*Clostridiales*	*Clostridiaceae*	0.0016	G	0.0207	M		
			Defluviitaleaceae	0.0215	G				
			Eubacteriaceae			0.0113	M		
			Lachnospiraceae	<0.0001	G	0.0029	M		
			Oscillospiraceae	0.0366	G			0.0089	G
	Erysipelotrichia			<0.0001	G			0.0476	G
		Erysipelostrichales		<0.0001	G			0.0194	G
			Eryspipelotrichaceae	<0.0001	G			0.0211	G
	Negativicutes			0.0015	G				
		Selenomonadales		0.0019	G				
			Veillonellaceae					0.0152	M
Proteobacteria	*Alphaproteobacteria*			0.0097	G				
	Betaproteobacteria	*Burkholderiales*	*Sutterellaceae*	0.0414	G				
	Deltaproteobacteria			0.0183	G			0.0087	G
		Desulfovibrionales		0.0366	G			0.0027	G
			Desulfovibrionaceae					0.0030	G
	Gammaproteobacteria	*Xanthomonadales*		0.0044	G				
			Xanthomonadaceae	0.0037	G				
Spirochaetes	*Spirochaetia*	*Spirochaetules*		0.0366	G			0.0256	G
			Spirochaetaceae					0.0244	G
Teniricutes	*Mollicutes*			0.0316	G				

Significantly high composition ratios ($P<0.05$) are shown in G, M, and F, for the grain-diet group, the meat-diet group, and the fish-diet group, respectively. Asterisks (*) indicate *P*-values that were close to reaching significance.

individual at the class level is shown in Fig. 5. No distinctive fluctuations in overall bacterial flora composition were observed for either diet groups at the class level, or the phylum, order, and family levels.

Compositional fluctuation: Some bacteria groups showed trends towards rough fluctuations (Fig. 6). At the Phylum level, a large increase in the ratio of *Actinobacteria* in the fish-diet group was observed once, around term 3, and was followed by a decrease. Nevertheless, the ratio of *Actinobacteria* still remained a high level compared with pre-treatment. On the other hand, the ratio of *Actinobacteria* in the meat-diet group remained the same, or decreased, when compared with pre-treatment ratios. Additionally, in term 7, the WTE individual in the fish-diet group with the swapped diet (fish2) had a decreased *Actinobacteria* ratio after being fed meat. Conversely, the *Actinobacteria* ratio for the individual in the meat-diet group given a swapped diet (meat2) increased in term 7.

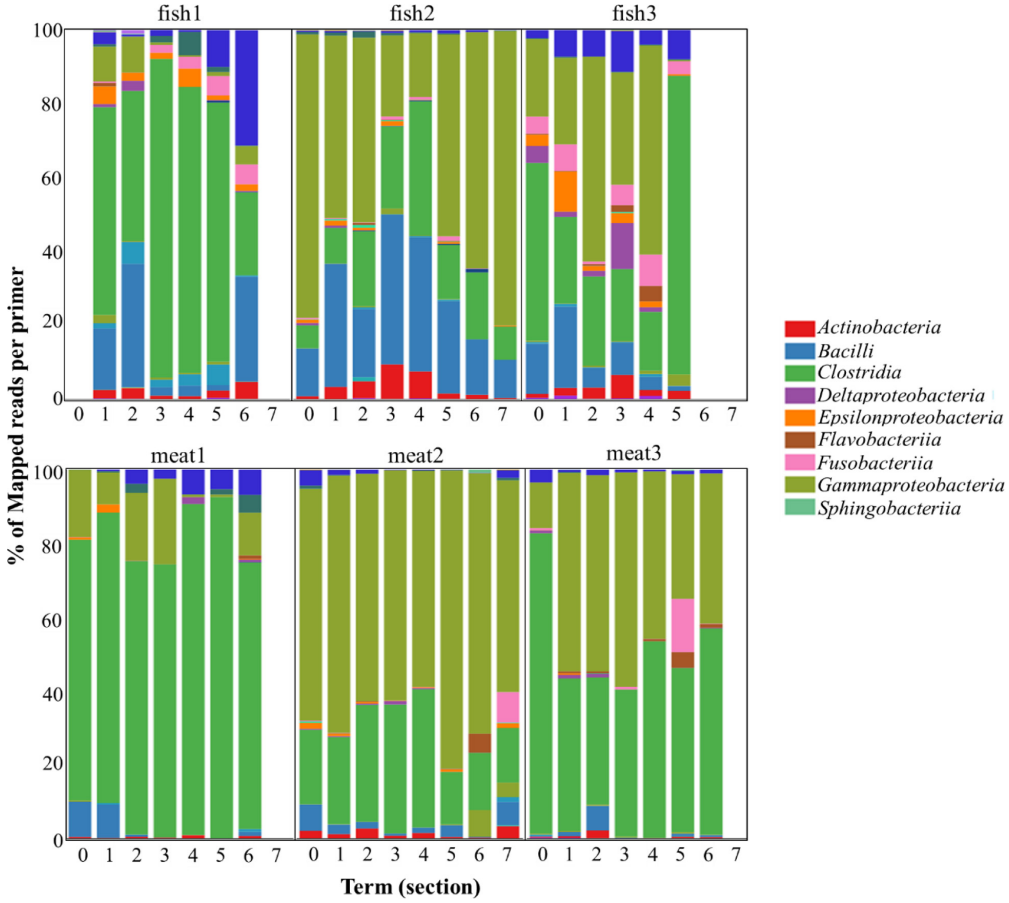

Fig. 5. Composition ratios of bacterial flora per term in each white-tailed eagle. The results show ratios at the class level. Term 7 shows results from the fish2 and meat2 individuals, and includes only the 33rd and 34th days of the swapped diet. Term 0 for fish1 and term 6 for fish3 are missing, because the corresponding samples could not be analyzed.

At the order level, the ratio of *Lactobacillales* in the fish-diet group consistently remained at a higher level than in the meat-diet group. Conversely, in the meat-diet group, the *Lactobacillales* ratio tended to decrease when compared with pre-treatment. However, the ratios of the two individuals with swapped diets that changed diet after Term 7 did not change much.

At the class level, the ratio of *Bacilli* was decreased in the meat-diet group. The ratio of *Bacilli* in the fish-diet group remained high during the one-month experimental period, but nevertheless decreased when compared to pre-treatment ratios, except in one individual. In addition to *Bacilli*, *Lactobacillales* and *Actinobacteria* also showed a trend where their ratios increased significantly at one point and then were somewhat reduced.

Coriobacteriales was found to gradually increase in proportion in the fish-diet group during the experiment period. There was no significant change in the ratio of *Coriobacteriales* in the meat-diet group.

The average composition of the gut microbiome in each group: Figure 7 shows the composition of bacterial flora in the control, meat-diet, and fish-diet groups. Different patterns of changes were observed in the fish-diet and meat-diet groups relative to the control group. At the family level, the ratio of *Lactobacillaceae* increased in the fish-diet group (17.50%) compared with the control group (8.78%) and the meat-diet group (0.74%). In addition, the ratio of *Clostridiaceae* decreased in the fish-diet group (10.40%) compared with the control group (28.73%).

Pasteurellaceae was higher in the meat-diet group (4.66%) than in the fish-diet group (0.37%). Whereas, *Veillonellaceae* levels were higher in the fish-diet group (10.52%) than in the meat-diet group (3.18%) in this study.

DISCUSSION

Comparison between avian species

As mentioned in Introduction, the gut microbiome plays essential roles in many ways, and is affected by various factors such as food habitat. In experiment 1, we tried to compare the diversity between species. α-Rarefaction analysis revealed that the grain-diet group had a wider variety of gut microbiome diversity than the other two groups (Fig. 2), which is in accordance with previous studies showing that herbivorous mammals have a high diversity of bacterial flora [15]. Herbivorous animals are thought to have a high diversity of bacterial flora because their diet comprises large amounts of non-digestible dietary fiber. In addition, they are also

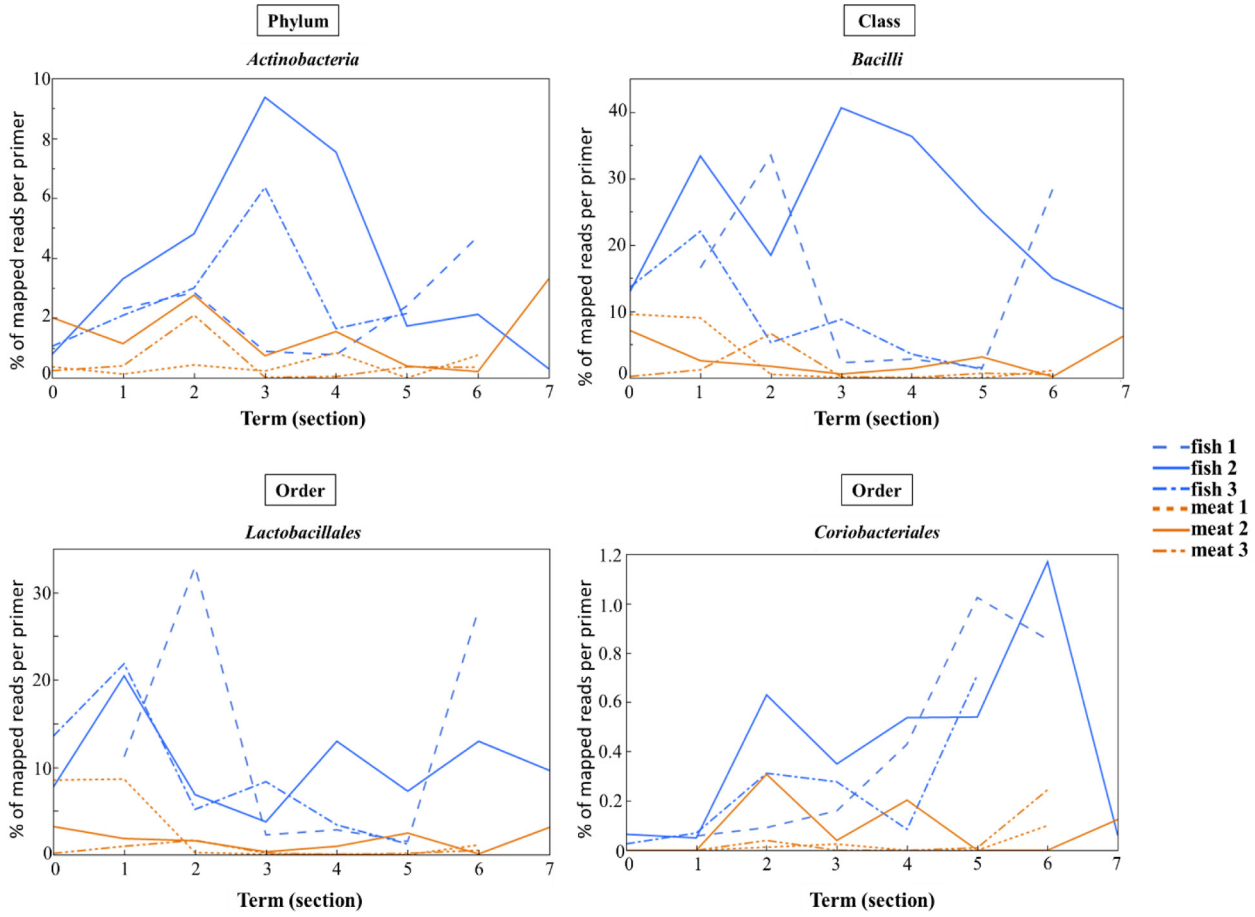

Fig. 6. Fluctuation trends in intestinal bacteria. The blue and brown lines show variation in the composition ratio of intestinal bacteria in the fish-diet group and the meat-diet group, respectively, in individual white-tailed eagles.

exposed to plant secondary compounds, which include toxic substances [9].

The calculation of Shannon index showed no significant difference between the grain-diet and the meat-diet groups (P=0.0853), whereas there was a significant difference (P=0.0017) between the grain-diet and the fish-diet groups (Fig. 3). This is likely caused by the high Shannon index for the great horned owl (3.4222), which was included in the meat-diet group. Generally, the cecum is long and developed in herbivorous birds, but is short or degenerated in carnivorous birds. Therefore, it is interesting to note that owls have a developed caecum, despite being carnivorous [18]. This may account for the high Shannon index observed in the great horned owl. In fact, when the great horned owl is excluded, the Steel-Dwass test shows a significant difference between the grain-diet and the meat-diet groups (P=0.0231).

From an unweighted UniFrac analysis, we clarified the rough trends based on diet (Fig. 4). Moreover, we also found the different plot positions depending on the host species and diet contents, even belonged at same diet groups. For example, the Indian peafowls, domestic geese, and ducks have the same diet, such as clover and oyster shells. These three species had similar bacterial flora, except for one individual. Ostriches also ate similar foods as the above species, such as oyster shells, but their microbiome composition differed. The ostrich may have a different microbiome composition due to its large cecum and relatively long body and intestinal tract. These findings reveal the strength of the influence of the diet and host strain factors on microbiome composition.

Gut microbiome composition differed between diet groups

As shown in Table 2, there were significant differences of bacteria species between the grain-diet group and the fish- and meat-diet groups. A significant difference was observed for *Bacteroidetes*, which degrade complex biopolymers and polysaccharides, such as carbohydrates and plant cell wall components, in the gastro-intestinal (GI) tract. The number of *Bacteroidetes* has been shown to decrease as a result of ingesting a high-fat diet [22]. Furthermore, *Bacteroidetes* primarily produce acetic and propionic acid as final metabolites, which could contribute to short-chain fatty acid (SCFA) production in herbivores. *Firmicutes* are known to be involved in SCFA production, and are especially involved in the production of butyric acid [8]. SCFA production from non-digestible dietary fiber may play an important role in the nutrition of herbivorous birds, because the proportion of many bacterial species was larger in the grain-diet group. For example, *Lactobacillaceae* are known to be involved in acetic acid production [11].

When focused on the difference between fish-diet and meat-diet groups, meat- diet groups showed higher composition than fish-diet

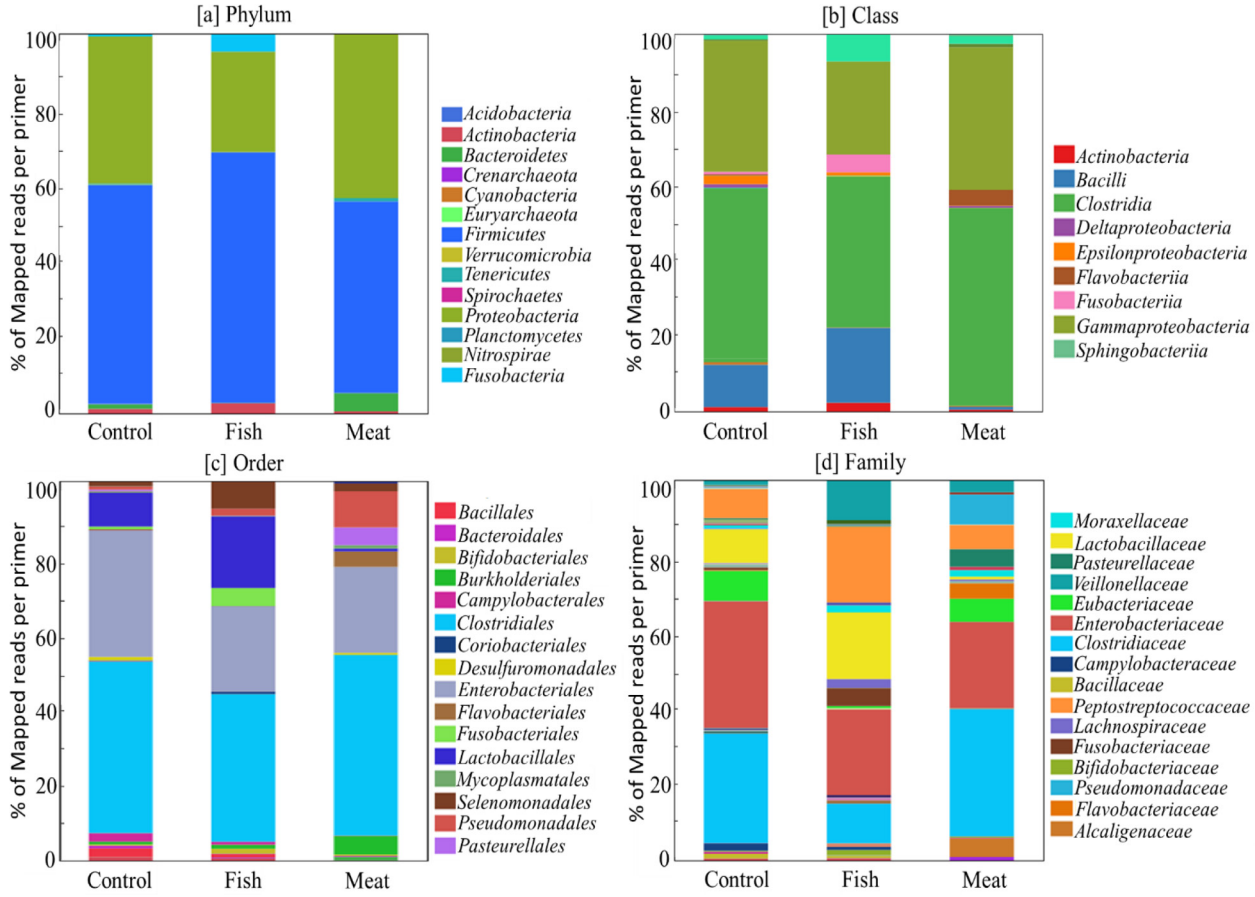

Fig. 7. The average composition of the gut microbiome in each group. Graphs showing the average composition of the intestinal flora in the control, fish-diet, and meat-diet groups at the: (**a**) phylum, (**b**) class, (**c**) order, and (**d**) family level. The control group was comprised of fecal samples collected before the white-tailed eagles were put on a specific diet. The control group also contained one individual white-tailed eagle that was not given the dietary treatment.

groups, especially *Veillonellaceae* (phylum: *Firmicutes*), *Eubacteriaceae* and *Lacnospiraceae* (both belonged to the *Clostridiaceae* family). As mentioned in the Introduction, SCFAs may be produced by fermenting cartilage and collagen, as opposed to non-digestible dietary fiber. The significant presence of these bacteria in the meat-diet group suggests that they may contribute to SCFA production in a carnivorous diet. Indeed, previous studies indicate that *Lachnospiraceae* and *Eubacteriaceae* are known as SCFA-producing and butyric-acid–producing bacteria,respectively [20, 21]. *Veillonellaceae* utilizes the partially degraded products of bacterial polysaccharides to produce acetic and propionic acid [10], and its ratio in kittens that ate moderate-protein, moderate-carbohydrate food has been shown to increase in comparison to kittens that ate high-protein, low-carbohydrate food [13]. It was concluded that the balance between the protein and the carbohydrate content of the diet affects the ratio of *Veillonellaceae*; therefore, the intestinal environment of the meat-diet group may be suitable for *Veillonellaceae*, compared with the intestinal environment of the grain-diet group. Additionally, *Veillonellaceae* likely contributes to SCFA production when the equilibrium of the intestinal flora that is characteristic of the meat-diet group is preserved.

In the fish-diet group, *Flavobacteriia* was the only bacteria class that was present in a significantly greater proportion than in the other two diet groups. *Flavobacteriia* is widely present in the GI tract, soil, and aqueous environments, among other areas [3, 22]. *Flavobacteriia* also comprises species that can become pathogens for birds and mammals, but it is still unknown how their increase influences the onset of disease.

Impact of fish diet on WTEs

The fish-diet group had a higher ratio of *Lactobacillales*, the order in which *Lactobacillaceae* (mentioned in experiment 1) belongs, than the meat-diet group. Since *Lactobacillaceae* are involved in the production of acetic acid, this observation suggests that acetic acid production may be increased by eating fish. Indeed, the ratio of *Lactobacillaceae* rose sharply when changing the meat diet of the meat2 individual to fish in term 7. Therefore, it can be said that *Lactobacillales* fluctuated due to the influence of the fish diet. Moreover, the same is true for *Actinobacteria* at the Phylum level. *Actinobacteria* include *Bifidobacteriaceae*, which produces a large amount of acetic and lactic acid. Lactic acid is very important because it is converted to butyric acid [4]. At the family level, the average ratio of *Bifidobacteriaceae* in the fish-diet group (1.36%) was higher than in the control (0.27%) or meat-diet groups (0.36%; Fig. 7d).

The increase in the ratio of *Actinobacteria* in the swapped-diet individual, meat2, in term 7 was likely caused by the change to the fish diet. Furthermore, in term 7, there was no change in the ratio of *Bacilli* in the fish2 individual, but the composition ratio in the swapped-diet individual in the meat-diet group (meat2) rose. This also indicates that compounds in the fish diet possibly promote *Bacilli* growth. There was no change in the ratios observed in the meat2 individual after the diet swap in term 7. However, the *Coriobacteriales* ratio was greatly reduced in the fish2 individual when it was given meat in term 7; therefore, it can be concluded that *Coriobacteriales* increased as a result of consuming fish. Much of the influence of *Coriobacteriales* is unknown, so it is difficult to speculate as to what such an increase means. Thus, we can only surmise that the fish-based diet provides suitable conditions for the growth of this bacterium.

Convergence of fluctuations in gut microbiome constituents

According to Fig. 6, we found some fluctuation trends in the ratio of *Bacilli*, and *Lactobacillales* and *Actinobacteria* as well. Considering these results, the intestinal microbial flora of WTEs may be more suited for a meat diet. If this hypothesis is correct, the meat diet may not cause sensitive fluctuations in bacterial flora, whereas the continuous feeding of fish, to which the intestinal flora do not adapt, could cause clear fluctuations. In this study, a continuous fish-only diet may have resulted in the gut microbiome of the fish-diet group adapting to their diet in around 10–20 days. Furthermore, as the fish-processing capacity of the bacterial flora gradually increased, the ratio of the bacteria which showed an increase would gradually fall.

As the fluctuation in each intestinal bacterium species should converge, the trend of an increasing *Coriobacteriales* ratio may be shown in the latter half of the one-month experimental period.

Integrated consideration of the insights from experiments 1 and 2

Through experiment 1 and 2, we could consider the diet dependent effects to WTEs. As mentioned in Result, there were different patterns of changes in the fish-diet and meat-diet groups relative to the control group (Fig. 7).

The tendency of increase of *Lactobacillaceae* in the fish-diet group compared with the control group and the meat-diet group was observed both in experiment 1 and 2 (Table 2, Fig. 7d). Furthermore, we also got the tendency of decrease of *Clostridiaceae* in the fish-diet group compared with the control group (Table 2, Fig. 7d). Similarly, the ratio of *Eubacteriaceae* was also significantly lower in the fish-diet group compared to the meat-diet group in experiment 1 (Table 2). Therefore, the observed specific diets had similar effects on gut microbiome composition in multiple avian species, were further confirmed among an allogeneic species.

Some bacteria species in the meat-diet group in experiment 2 showed characteristic changes that were not found in experiment 1. For example, in experiment 2, *Pasteurellaceae* was higher in the meat-diet group than in the fish-diet group (Fig. 7d). Dong *et al*. [10] examined the relationship between fecal microbiota and SCFA concentration in children with or without cow milk protein allergies. They found that *Pasteurellaceae* was correlated with the concentration of propionic acid, suggesting that the levels of propionic acid production may be high in the meat-diet group. Indeed, in experiment 2, *Pseudomonadaceae* was found in higher levels in the meat-diet group compared with the fish-diet group. The *Pseudomonadaceae* family has been found to cause infectious diseases in some birds [14]. Likewise, *Pseudomonadaceae* is related to inflammatory enteritis in humans, and its toxins cause damage to epithelial cells. It remains unclear how the composition of the Pseudomonadaceae family increases, and whether such an increase has any meaningful effects.

In experiment 1, the average *Veillonellaceae* ratios in the meat and the fish-diet groups were almost the same, but this ratio was higher in the fish-diet group than in the grain-diet group (Table 2). Whereas, in experiment 2, the levels of *Veillonellaceae* differed between in the fish-diet group and the meat-diet group (Fig. 7d). As described in experiment 1, it is likely that the ratio of protein and carbohydrate content of the dietary components influences *Veillonellaceae* proportion. Accordingly, it follows that the intestinal environment that is generated by a fish diet appears to be most suitable for *Veillonellaceae*. *Megasphaera*, which belongs to the *Veillonellaceae* family, is a major butyric acid-producing bacterium; therefore, the prosperity of the *Veillonellaceae* family is an important factor in identifying influences on the intestinal barrier [2].

Limitations of experiment 2

There were some differences in the gut microbiome composition between the control group and the two diet groups, but these were not significant. Two factors may have contributed to these observations. First, the number of individuals in each group was small, and fluctuation strength varied depending on differences in individual bacterial flora. Thus, identifying significant differences could be difficult. Second, the WTEs were not force-fed, so the amount of food that they consumed varied daily. This could account for the fact that similar trends in fluctuation were not observed for each period. Therefore, it is necessary to repeat the research to increase the number of samples collected and verify these observations. In addition, it is important to optimize the experimental method, for example by ensuring uniformity in the amount of food consumed.

Conclusion

We investigated influences of diet on the composition of the gut microbiome using the feces of bird species. The gut microbiome compositions of the meat and the fish-diet groups were similar when compared with the grain-diet group. The grain-diet group had higher gut microbiome diversity than the meat- and fish-diet group. The composition of the intestinal microflora differed between the fish and meat diet groups, even for the same animal species, and an increase in bacterial composition specific to the meat diet group was observed, indicating that the composition of the intestinal microflora differed depending on the diet.

CONFLICT OF INTEREST. The authors declare there are no conflicts of interest.

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