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Are microbes and metabolites influencing the parental consumption of nestlings' feces in gray-backed shrikes?

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Abstract

The behavioral video recordings of the gray-backed shrike *Lanius tephronotus* revealed that parent birds eat the feces produced by their nestlings. "Parental nutrition hypothesis" attributes the origin of this behavior to nutrition-recovery and cost-saving, respectively. However, the presence of usable nutrients in the nestlings' feces is unknown because of traditional technology. In this study, we analyzed all the metabolites and the variations in the diversity and content of microbes in the feces of gray-backed shrike nestlings. We aimed to report the changes in microbes and metabolites with the age of nestlings and point out that the parent birds that eat the feces may gain potential nutrition benefits. The results showed that the relative abundances of Proteobacteria, Firmicutes, and Bacteroidota, changed significantly when the nestlings were 6 days old. The relative abundances of 6 probiotics, which are involved in digestion, metabolism, and immunity-related physiological functions, decreased in the nestlings' feces gradually with age; therefore, these probiotics may be obtained by parent birds upon ingestion of the feces of young nestlings. Among the metabolites that were detected, 20 were lipids and some had a role in anti-parasitic functions and wound healing; however, their relative contents decreased with age. These beneficial substances in the nestlings' feces may stimulate the parents to swallow the feces. Moreover, there were many aromatic metabolites in the newly hatched nestlings' feces, but the content of bitter metabolites increased as they grew up. Therefore, our results are in accordance with the nutritional hypothesis.

Key words: feces disposal, metabolite, microbiota, nestlings, probiotics.

Birds have evolved a series of behavioral strategies to adapt to dynamic environments, improve the survival rates of their offspring, and reduce their own losses (Lima 1986; Pappas et al. 2010). It is common for parent birds to dispose the feces of their offspring (Dell'Omo et al.1998). This behavior is considered to be of positive significance for breeding ecology because it can maintain the sanitation of the nest environment (Thomson 1934; Spencer 2005); hence, this reduces the risk of nest predation, and bacterial infection in nests improves the survival rate of the nestlings (Lang et al. 2002; Ibáñez-Álamo et al. 2014; Azcárate-García et al. 2019).

The nestlings' feces are usually wrapped in fecal sacs, and there are 2 behavioral strategies adopted by birds to dispose fecal sacs, namely, direct ingestion of the fecal sacs just after the nestlings produce them in the nest, or flying away with the fecal sacs and disposing them far away from the nest (Witherby 1924; Petit et al. 1989). Previous studies have shown that parent birds can adopt both strategies; and they are more prone to swallow their offsprings' feces during the early nestling period, but later, as nestlings aged, they are more likely to dispose the fecal sacs (Morton 1979; Alberts and Gubernick 1983). For example, in case of goldfinches *Carduelis carduelis*, the tree swallows

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Tachycineta bicolor and the red-winged blackbirds *Agelaius phoeniceus*, when the nestlings are >7 days old, the parent birds stop eating their feces and use a completely different strategy which is they discard the feces (Glück, 1988; Hurd et al.1991).

The fecal consumption behavior is not limited to birds; it also observed in vertebrates like plateau pikas *Ochotona curzoniae* (Alberts and Gubernick 1983; Speakman et al. 2021). Different theories have attempted to explain this interesting behavior of parent birds. The "parental nutrition hypothesis" suggests that the digestive system of young nestlings is not perfectly developed; therefore, many nutrients remain in the nestlings' feces (Dell'Omo et al.1998). Parent birds eating feces can supplement the energy required for different activities during this period. However, as the offsprings grow, their digestive systems develop completely, and they can fully digest their food (Morton et al. 1972). Consequently, the parent birds stop consuming their nestlings' feces and discard them instead (Dell'omo et al. 1998). Previously published papers have discussed the nutritional composition of the nestling' fecal matter with respect to the water content, energy content, and carbohydrate density of the nestlings' fecal matter (Skadhauge 1981). However, these discussions are not enough to understand the nutritional significance of feces to birds not just because it has been observed that a bird ingested feces to obtain vitamins (Negro et al. 2002), but also because of the important effects of fecal microorganisms on animal nutrition (Aziz et al. 2013; Lindsay et al. 2020). It is difficult to identify these components and examined their changes in the feces by traditional technology; therefore, the nutritive value obtained by parent birds upon consumption of the nestlings' feces at different day–ages is unknown. With the untargeted metabolomics development, we can get the complex composition of feces that includes the nestlings' metabolites and some microbial metabolites. Quantitative analysis of metabolites in the feces of nestlings at different day–ages can help to accurately understand the changes in the fecal nutrient levels and the related metabolic pathways. The 16S rRNA amplicon sequencing technology has improved the identification of low abundant microbial species, thus improving the integrity of microbial community research. These technologies can help understand the dynamic changes in fecal composition with age.

The gray-backed shrike *Lanius tephronotus* is a bird that inhabits the Qingzang Tibet Plateau, and commonly portrays biparental care (Lu et al. 2010). The food preferred by these birds remains unchanged throughout the nestling period. However, video recordings revealed fecal matter swallowing behavior in both male and female gray-backed shrikes. They maintain a high frequency of fecal swallowing when the nestlings are young and suddenly stop this behavior when the nestlings grow to around 7 days old. In this study, the fecal microorganisms and metabolites of gray-backed shrike nestlings were analyzed at different ages, and the observed differences were compared. The aim was to point out that the parent birds that eat the feces may gain potential nutrition benefits. Here, we report the changes in the microbial compositions of the feces of gray-backed shrike nestlings. We expected the probiotic content in nestling feces to decrease with the growth of nestlings. Untargeted metabolomics was first used to identify the composition of bird feces. We expected the young nestlings' feces to contain a high amount of beneficial metabolites. We also assessed that the potential benefits and functions of the probiotics and beneficial metabolites to parent birds.

Materials and Methods

Study site and species

The research site was in Luqu County (102°05′ E–102°47′39″ E; 33°58′12″ N–34°32′16″ N) of the Gannan Autonomous Prefecture, Gansu Province, China. The gray-backed shrike, which is one of the most common birds in the Qingzang Tibet Plateau (Lu et al. 2010), was selected as the research organism. These birds often nest in the alpine shrubs, which consist of barberries and sea buckthorns, and they reproduce once a year with 3–5 eggs in the nest. Their breeding season generally begins in mid-May and ends in early August. Compared with other shrike species, the survival rate of gray-backed shrike nestlings is higher. It takes ~20 days for the eggs to hatch, and the offspring are usually fed on insects by their parents; they experience biparental care and leave the nest 13–15 days after hatching (Zack 1986). During the breeding period, the parent birds face fewer health risks, owing to the richness of food resources and mate choice. However, they face high nest predation risks. According to our recording, 100% of nest failures occur because of predators, such as oriental magpie *Pica serica* and steppe polecat *Mustela eversmanii*. The parent birds spent more time defending the nest when the nestlings were young than when the nestlings grew older.

Sample collection

Between May and August 2020, we randomly selected gray-backed shrike nestlings ($n = 29$) from 13 nests as our sampling organisms. To reduce the human nest interference, we started collecting feces from the second day after the nestlings hatched and continued to collect feces every 2 days until the nestlings left the nest. Finally, 131 fecal samples were obtained, including 8 complete sets of samples. A complete set refers to fecal samples of the same nestlings collected at the ages of 2, 5, 8, 11, and 14 days. Three complete sample sets were used for 16S rRNA analysis, and the remaining 5 sets were used for metabolite analysis. The remaining 91 samples were not include in the analysis because we could not obtain feces from the same individuals at regular intervals due to the high mortality of nestlings and the inability to reach the sampling point due to extreme weather. During fecal sample collection, we placed the nestlings on our palms, wiped the skin near their cloaca with a cotton head dipped in 75% alcohol, and positioned the sterilized cryopreservation tube just outside the cloaca of the nestlings. Thereafter, we gently shook the nestlings and waited for them to defecate. The fecal collected samples as soon as the nestlings released the feces and placed the cryopreservation tubes into the icebox after marking them. All samples were initially stored in 2 mL sterilized cryopreservation tubes at −20 °C and finally transferred to a − 80 °C refrigerator after arriving at the laboratory. It should be noted that the duration for shaking the nestlings was related to their age. The skin of 1- to 5-day-old nestling is thin. If the nestling was about to defecate, the feces in the cloaca could be directly observed. Shaking the nestlings for ~10 s would be sufficient to release the feces. Because the cryopreservation tube was directly connected to the cloaca of the nestling, the feces were excreted directly into the cryopreservation tube. This process did not require any fecal transfer tools, so the contamination of the sample with environmental microbes during sample collection was avoided. Nestlings that were over 5 days old naturally defecated after shaking for up to 30 s after disinfection. Nestlings that did not defecate even after shaking for more than the 30 s were deemed to have no urge to defecate, so stool sampling was stopped, and the nestlings were put back into the nest.

DNA extraction and 16S rRNA gene amplification and sequencing

Genomic DNA was extracted from the fecal samples by the CTAB method, because this method has applicability on universal

experimental objects and can effectively eliminate the interference of food residue in feces to improve the amount of DNA extraction. The purity and concentration of the extracted DNA samples were detected on 1% agarose gel. Thereafter, the DNA samples were placed in centrifuge tubes and diluted with sterile water to a final concentration of 1 ng/μL. The 16S V3–V4 regions of the 16S rRNA gene were amplified with appropriate primers (341F: CCTAYGGGRBGCASCAG; 806R: GGACTACNNGGGTATC TAAT); all polymerase chain reactions (PCRs) were carried out using 15 μL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA). About 2 μM of forward and reverse primers, and about10-ng template DNA. The thermal cycle process includes initial denaturation (98 °C) for 1 min, followed by 30 cycles of denaturation for 10 s (98 °C), annealing for 30 s (50 °C), elongation for 30 s (72 °C). Finally, kept for 5 min (72 °C). The amplified products were detected by a 2% agarose gel electrophoresis. The PCR products were mixed in an equal density ratio, and the resultant PCR product mixture was purified with Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany). The DNA library was generated using TruSeq DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA), and index codes were added to attribute sequences to each sample. The quality of the constructed library was assessed using the Qubit@ 2.0 Fluorometer (Thermo Scientific, Waltham, MA) and Agilent Bioanalyzer 2100 system. Finally, the library was sequenced using the NovaSeq platform, and 250-bp paired-end reads were generated. According to the unique barcodes of the samples, the paired-end reads which are assigned to the sample and truncated by cutting the barcode and primer sequence were merged using FLASH (version 1.2.7; Magoč and Salzberg, 2011). Raw tags obtained by splicing had to be strictly filtered to get clean tags. We obtained high-quality clean tags (Bokulich et al. 2013) from the raw tags based upon the QIIME (version 1.9.1; Caporaso et al. 2010) quality-controlled process, which was performed under specific filtering conditions. We used the Silva database to compare the tags and UCHIME algorithm to detect and remove chimaera sequences (Edgar et al. 2011; Haas et al. 2011) and finally obtain the effective tags. Sequence analysis was performed using the Uparse software (version 7.0.1001) (Edgar, 2013), and the sequences with ≥97% identity were assigned to the same operational taxonomic unit (OTU). Taxonomic information, which was annotated using the Silva Database (http://www.arb-silva.de/) (Quast et al. 2013), was used based on the Mothur algorithm. And the multiple sequence alignment was conducted for phylogenetic relationship construction using the MUSCLE software (version 3.8.31; Edgar, 2004). The OTUs abundance information was normalized using a standard sequence number corresponding to the sample with the least sequences. Microbes were identified at phylum and genus levels.

Fecal metabolite extraction and ultra-high-performance liquid chromatography–mass spectrometry analysis

Metabolites were extracted from 35 fecal sample bottles according to previously reported conventional methods (Barri and Dragsted 2013; Want et al. 2006). Hundred microliter of the fecal sample was taken in an EP tube, resuspended with pre-chilled 80% methanol and 0.1% formic acid, and mixed well by vortexing. Subsequently, the EP tube was subjected to vortex oscillation and placed in an ice bath for 5 min, after centrifugation (15,000 *g*, 4 °C, 20 min). After incubation, 40 μL of the supernatant was collected and diluted to 53% methanol content using mass spectrometry grade water, transferred to a new EP tube, and centrifuged again (15,000 *g*, 4 °C, 20 min). Finally, the supernatant was injected into the liquid chromatography–tandem mass spectrometry system for analysis. The same volume of experimental sample was taken for the quality control (QC) samples, and the experimental sample was replaced by 53% methanol/water solution for the "blank sample," in which the pre-treatment process was the same as that of the experimental samples. We performed the ultra-high-performance liquid chromatography–tandem mass spectrometry analyses using a Vanquish UHPLC system (ThermoFisher, Hennigsdorf, Germany) coupled with an Orbitrap Q ExactiveTM HF mass spectrometer. Mass spectrometry conditions (including the canning range): *m/z* 100–1,500, spray voltage: 3.2 kV, sheath gas flow rate: 40 arb, auxiliary gas flow rate: 10 arb, capillary temperature: 320 °C.

Data processing and metabolite identification

The raw data files were imported into Compound Discoverer (version 3.1) for the preliminary screening of the retention time, mass/charge ratio, and other parameters to quantify the metabolites. The main parameter settings for this process were the retention time deviation (0.2 min), the mass deviation (5 ppm), signal intensity tolerance (30%), minimum intensity (100,000), and signal/noise ratio (3%). After that, the molecular formulae of the metabolites were predicted based on the molecular ion peaks and fragment ions and compared with mzCloud (https://www.mzcloud.org/), mzVault, and MassList databases. The priority of metabolite identification results is mzCloud > mzVault > Masslist. The background ions were removed with blank samples, and the quantitative results were normalized with the QC samples. Finally, the identification and quantitative results were obtained from the data, and the obtained metabolite data were further processed by statistical R (version 3.4.3), Python (version 2.7.6), and CentOS (version 6.6).

Statistical analyses

The alpha diversity index of community biodiversity, including the number of OTUs per sample, Chao1 index, Shannon index, Simpson index, and Abundance-based Coverage Estimator (ACE) index, were calculated with QIIME (Caporaso et al. 2010) and displayed with the R (version 2.15.3). We also used R (version 2.15.3) to analyze the differences in alpha diversity index among the different groups and draw the rarefaction curve and rank abundance to aid comparisons of alpha diversity. The difference between groups of alpha diversity index was analyzed by t test, Tukey's test, and Wilcox test of agricola package. Moreover, the beta diversity index of community biodiversity was calculated on both weighted and unweighted Unifrac using the QIIME (version 1.9.1). Cluster analysis was preceded by principal component analysis (PCA) using the "FactoMineR" package, and the "ggplot2" package in R (version 2.15.3) was used to analyze the differences among the groups of beta diversity index. Parametric and nonparametric tests, that is, the Tukey's test and the Wilcox test, respectively, were carried out using the "agricola" package in R (version 2.15.3). The ANOSIM test was conducted using "vegan" package for analyzing the significance of community structure differences between groups.

Metabolonomics data were compared with the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/), Human Metabolome Database (http://www.hmdb.ca/), and LIPIDMaps database (http://www.lipidmaps.org/). The PCA and partial least squares discriminant analysis were performed in the metaX software (Wen et al.2017). The *t* test was applied to calculate the statistical significance (*P*-value) in R (version 3.4.3). The metabolites with Variable

Importance in the Projection > 1, $P < 0.05$, and fold change (FC) ≥ 2 or FC ≤ 0.5 were defined as the differential metabolites. Volcano plots drawn by "ggplot2" package in R (version 3.4.3) were used to filter metabolites of interest, and was based on log2 (FoldChange) and −log10 (*P*-value) of metabolites. The correlation between differential metabolites was used analyzed by "cor()" in R(version 3.4.3). The significance of the difference between metabolites was calculated by "cor.mtest()," and correlation plots were plotted by "corrplot" package in R (version 3.4.3), $P < 0.05$ was considered as statistically significant. For obtaining clustered heat maps, the data were normalized by the *Z*-scores, and the clustered heat maps were drawn with the "pheatmap" package in R (version 3.4.3). The functions and metabolic pathways of the metabolites were obtained from the Kyoto Encyclopedia of genes and genes (KEGG) database, which contains multiple databases. KEGG is categorized in terms of building blocks in the genomic space and wiring diagrams of interaction and reaction networks, known as KEGG PATHWAY. KEGG PATHWAY database is a collection of metabolic pathways and used to annotate the identified metabolites. The main biochemical metabolic pathways and signal transduction pathways involved by metabolites can be determined by pathway analysis. If the ratio condition $x/N > y/N$ is satisfied, the pathway is the KEGG enrichment pathway. x: The number of differential metabolites associated with the pathway; y: The number of all background metabolites associated with this pathway; N: the number of differential metabolites annotated by KEGG; N: the background of KEGG annotation, the number of all metabolites. With the help of the hypergeometric test method, the *P*-value of pathway enrichment is obtained, when the $P < 0.05$ is taken as the threshold, and the KEGG pathway meeting this condition is defined as the KEGG pathway significantly enriched in different metabolites.

Results

Gut microbiota profiles of gray-backed shrike nestlings

A total of 1,062,933 high-quality 16S rRNA gene sequences were obtained after DNA extraction from 15 fecal samples. To study the microbial species composition of each sample, OTU clustering was performed on all valid tags with 97% identity. We identified and annotated 8,416 OTUs, and they were divided into 17 phyla, 120 classes, 246 orders, 333 families, and 510 genera. The results showed that there were significant differences in the microbiota composition at the individual level and even among individuals of the same age. The number of OTUs of intestinal microorganisms was highest in the 14-day-old nestlings (838) followed by those in 8-day-old nestlings (541), and the number of OTUs of intestinal microorganisms was the lowest in 5-day-old nestlings (253). The number of unique OTUs was highest in the 14-day-old nestlings (596) and least in the 5-day-old ones (74; Figure 1A). The α diversity index of intestinal microorganisms was highest in 14-day-old nestlings and lowest in 5-day-old nestlings (Table 1, Supplementary Figure S1, and Supplementary Material S1). Based on the Principal Co-ordinates Analysis analysis, there were significant differences in the intestinal microbial composition among different gray-backed shrike nestlings, even of the same age (Figure 1B,C).

Changes in intestinal microbiota composition at different ages of gray-backed shrike nestlings

According to the results of species annotation, the 10 most abundant species in the intestinal microbiota of the gray-backed shrike nestlings, at the phylum level, at different day–ages were selected to generate a column accumulation diagram denoting the relative abundance of the species (Figure 2A). It was observed that the relative abundances of Proteobacteria and Firmicutes were comparatively higher than those of other bacterial phyla on each day. The relative abundance of Firmicutes increased as follows: Day 2 (14.09%), Day 5 (59.27%), and Day 8 (64.17%); its abundance was the highest in 8-day-old nestlings, and it began decreasing on Day 11 (13.17%) and Day 14 (3.49%). The relative abundance of Proteobacteria began to decrease as follows: Day 2 (46.87%), Day 5 (36.83%), and Day 8 (9.41%); however, its abundance increased significantly on Day 11 (84.74%) and Day 14 (79.70%) nestlings. The abundance of Proteobacteria was much higher than that of the other microorganism groups at 11 and 14 days of age. The relative abundances of the top 10 intestinal microorganisms of the gray-backed shrike nestlings, with highest abundance at the genus level, were recorded at different ages (Figure 2B). The results showed that *Rickettsiella* was the dominant intestinal bacterium in the nestlings at the age of 2, 5, 11, and 14 days, but its relative abundance was low in 8-day-old nestlings; additionally, it was observed that *Candidatus Arthromitus* formed the dominant bacteria in the 8-day-old nestlings. This suggests that the intestinal environment of 8-day-old nestlings may have undergone certain changes. The relative abundances of all the identified intestinal microorganisms were compared along the time period. The relative abundances of *Lactobacillus*, *Rombotusia*, *Turicibacter*, *Allobaculum*, *Ruminococcus gnavus* group, *Bifidobacterium*, and *Faecalitalea* decreased significantly with the increase in the nestlings' age, while the relative abundances of *Agathobacter* and *Brevundimonas* increased with age (Figure 3).

Profiles of fecal metabolites of gray-backed shrike nestlings

Based on high-resolution mass spectrometry, 734 metabolites were identified and annotated in the nestlings' feces. We analyzed the functional pathways with which these metabolites were involved, and it was observed that most of them were commonly involved in metabolism. After PCA, significant differences were observed in the metabolite composition of the feces of nestlings at different day–ages, and this difference decreased at the age of 12 and 14 days (Figure 4).

Changes in fecal metabolite composition at different day–ages of the gray-backed shrike nestlings

We screened the fecal metabolites of the nestlings along the time period, and we observed that the composition of the fecal metabolites varied greatly with the development of the nestlings. There were significant differences between the fecal metabolite compositions of 2- and 5-day-old gray-backed shrike nestlings. We identified 60 metabolites with significant differences, among which 16 were significantly upregulated and 44 were significantly downregulated (Figure 5 and Supplementary Material S2). The results of the pathway enrichment analysis of differentially expressed metabolites showed that these metabolites have significant effects on amino acid biosynthesis (Figure 6). Moreover, there were significant differences between the fecal metabolite compositions of 5- and 8-day-old

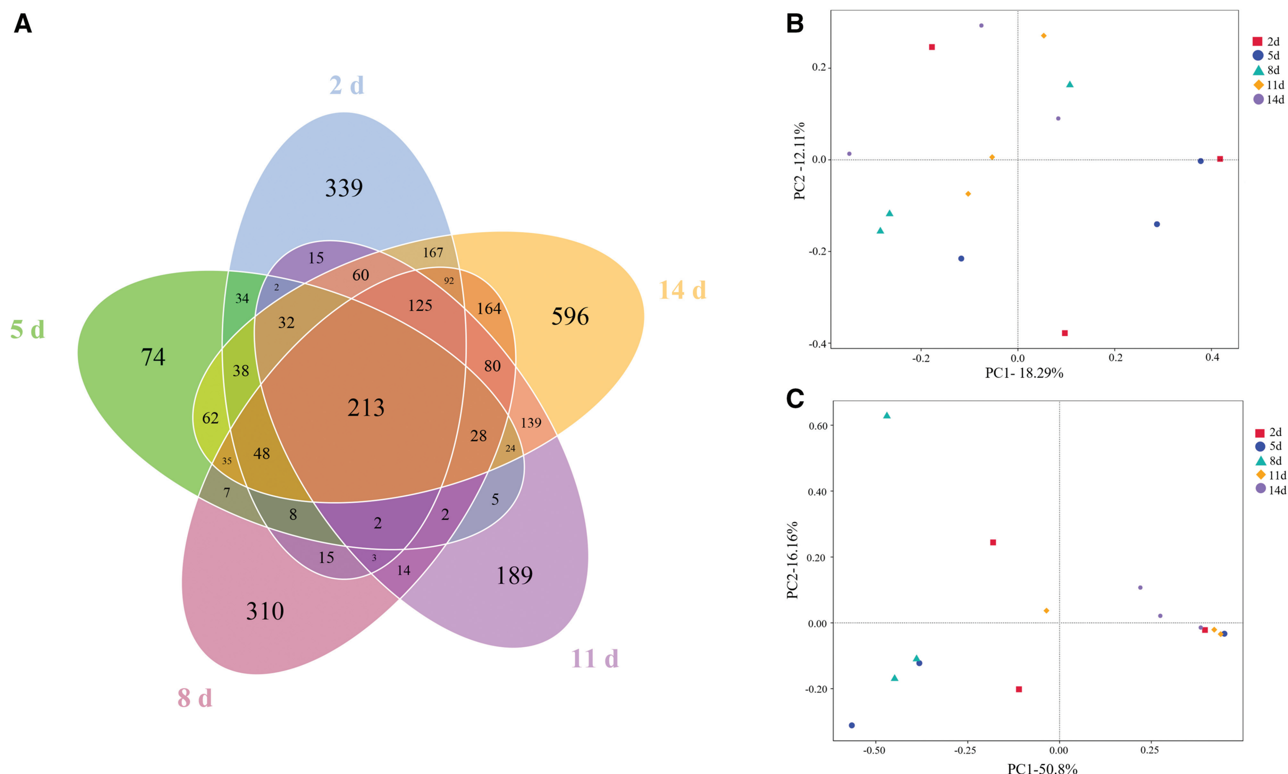

Figure 1. Microbial diversity in feces of nestlings on 2, 5, 8, 11, and 14 day–ages. (**A**) Each circle in the figure represents a group sample of the gray-backed shrike nestlings' feces on 2, 5, 8, 11, and 14-day–ages, the numbers of circles and overlapping parts of circles represent the number of OTUs shared between groups, and the numbers without overlapping parts represent the number of unique OTUs of the group. The number of OTUs shared by nestlings at all day–age stages was 213. The number of unique OTUs was the highest in 14-day-old nestlings is 596 and was the least in the 5-day-old ones is 74. (**B**) Analysis of similarities among fecal microbial communities of nestlings on 2, 5, 8, 11, and 14-day–ages based on unweighted Unifrac distance principal coordinate analysis (PCoA). There were significant differences in the intestinal microbial composition among different gray-backed shrike individuals. (**C**) Analysis of similarities among fecal microbial communities of nestlings on 2, 5, 8, 11, and 14 day–ages based on weighted Unifrac distance (PCoA). There were significant differences in the intestinal microbial composition among different gray-backed shrike individuals.

Table 1 Alpha diversity indexes of gut microbiota of gray-backed shrike nestlings

Group, days	Observed species	Shannon	Simpson	chao1	ACE
2	464	2.839	0.570	496.933	501.335
5	253	1.443	0.276	276.252	277.218
8	541	2.992	0.593	606.788	620.513
11	437	1.125	0.285	515.840	534.517
14	838	3.409	0.577	892.905	902.571

The α diversity analysis index of fecal microorganisms of gray-backed shrike nestlings was statistically analyzed at 2, 5, 8, 11, and 14 day–ages.

nestlings. We identified 87 metabolites, among which 40 were significantly upregulated and 47 were significantly downregulated (Figure 5 and Supplementary Material S2), and the pathway enrichment analysis of these differentially expressed metabolites showed that they mainly affect retinol metabolism (Figure 6). Furthermore, there were significant differences between the fecal metabolite compositions of 5- and 11-day-old nestlings wherein we identified 102 metabolites with significant differences, among which 55 metabolites were significantly upregulated and 47 metabolites were significantly downregulated (Figure 5 and Supplementary Material S2). Pathway enrichment analysis showed that these differentially expressed metabolites had significant effects on caffeine and retinol metabolism (Figure 6). The difference between the fecal metabolite compositions of 11- and 14-day-old nestlings was smaller; there were only 56 metabolites with significant differences, among which 45 were upregulated and 11 were downregulated (Figure 5 and Supplementary Material S2). Pathway enrichment analysis of these differentially expressed metabolites showed that they had significant effects on steroid hormone biosynthesis (Figure 6).

The results of Z-score analysis showed that maximum change in the numbers of differentially expressed metabolites was observed when the fecal metabolite compositions of 8- and 11-day-old nestlings were compared (Figure 7). It was observed that when the nestlings are 2, 5, and 8 days old, the parent birds ingested their offsprings' feces; however, the parent birds gradually stopped consuming their nestlings' feces around Days 11–14. The differences in the fecal metabolite compositions between the fecal ingestion stage and the fecal discarding stage were analyzed, and it was observed that 5 metabolites were significantly upregulated and 11 were significantly downregulated (Table 2). Additionally, a comparison between the relative contents of fecal metabolites in the nestlings that were just about to leave their nests and the ones that had just hatched showed that 60 metabolites, such as LPE 15:0, acetoacetate, indole-2-carboxylic acid, pantethine, rotenone, allantoin, alpha-benzylsuccinic acid, and robenidine, were significantly lower in the former group. On the contrary, the relative contents of 42 metabolites, namely 3-hydroxysebacic acid, 2, 4-Dichlorophenol, 2-amino-adipic acid, and catechin, were significantly higher in the feces of the

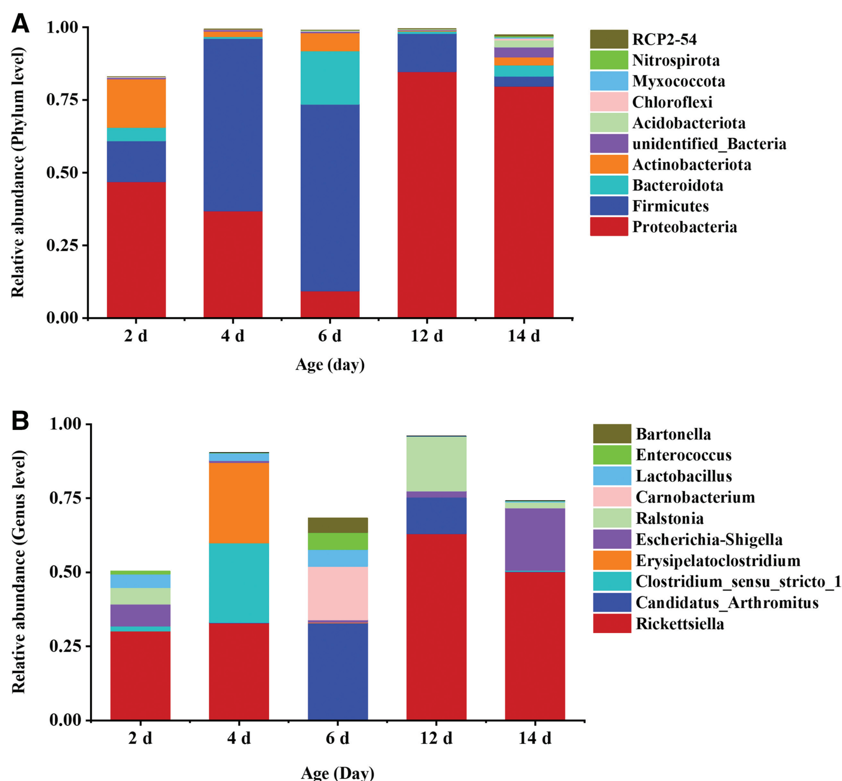

Figure 2. Relative abundances of the 10 most abundant species of microorganisms in feces of gray-backed shrike nestlings at 2, 5, 8, 11, and 14 day–ages at phylum and genus level. (**A**) Relative abundances of microorganisms in feces of nestlings on 2, 5, 8, 11, and 14 days at phylum level. (**B**) Relative abundances of microorganisms in feces of nestlings on 2, 5, 8, 11, and 14 days at genus level. Statistical analysis was performed by Student's *t*-test. $*P < 0.05$.

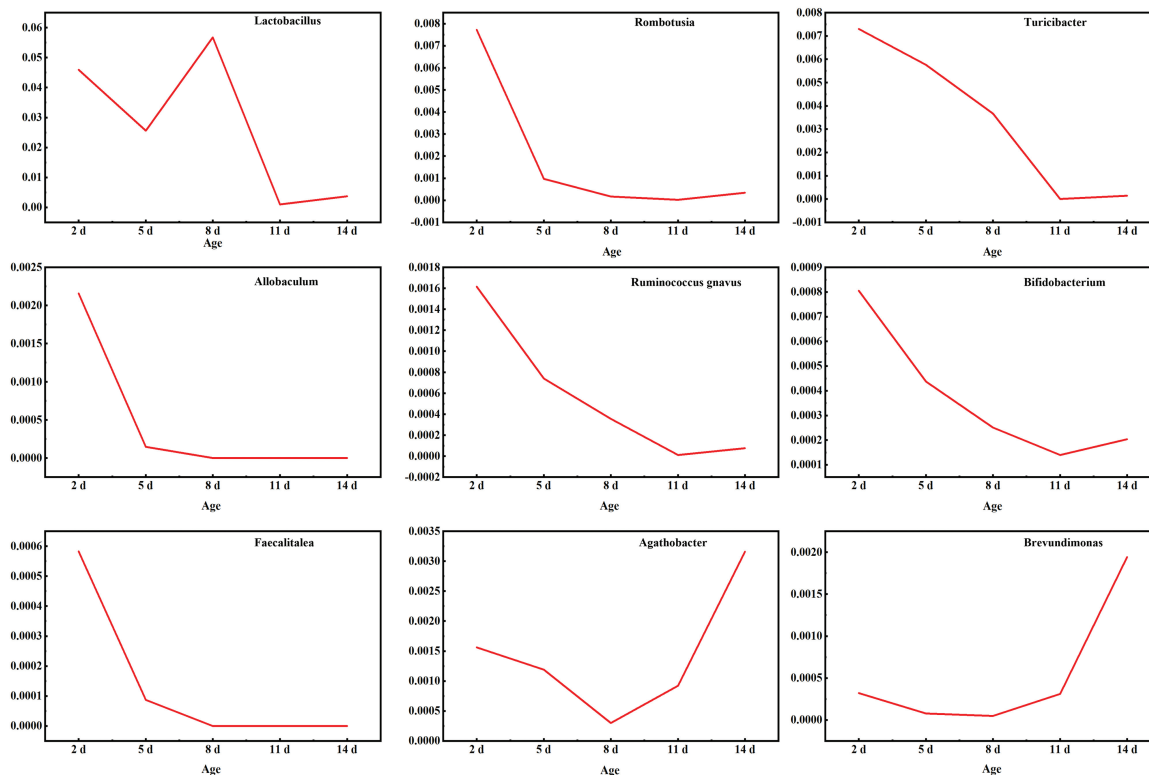

Figure 3. Variation trend of relative abundances of fecal microorganisms of gray-backed shrike nestlings. With the growth of the nestlings, the relative abundances of 7 microorganisms (*Lactobacillus, Rombotusia, Turicibacter, Allobaculum, Ruminococcus gnavus group, Bifidobacterium, and Faecalitalea*) decreased and that of 2 microorganisms (*Agathobacter and Brevundimonas*) increased.

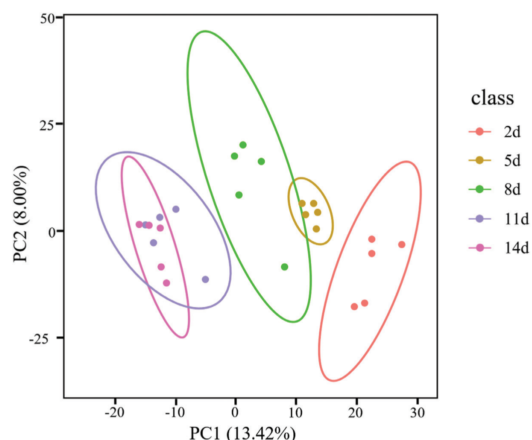

Figure 4. PCA analysis established the relationship model between metabolite expression and feces samples of nestlings on 2, 5, 8, 11, and 14 days to predict the metabolite difference in the samples of gray-backed shrike nestlings. Metabolites differ significantly in the gray-backed shrike nestlings' feces on 2, 5, 8, 11, 14 day–ages, but this difference weakens at 11 day–ages.

nestlings that were about to leave the nest than in the ones that were just hatched.

Discussion

It has been observed by video (11,040 min) among the gray-backed shrikes (Supplementary Material S3) that the parent birds consume their nestlings' feces with high frequency (70–100%) for a few days just after they hatch (Supplementary Figure S2 and Supplementary Material S1). However, about a week later, the parental behavior of ingesting the offsprings' feces stops; instead, they begin to discard their nestlings' feces far away from the nest (Supplementary Figure S2 and Supplementary Material S1).

We found that both microbial composition and community structure changed in the feces with the age of nestlings. The microbial alpha diversity showed no significant differences during the nestlings' growth period, but it was much higher in the feces of 2-day-old nestlings was much higher than that of 5-day-old nestlings (Table 1 and Figure 1A). The microbial β diversity of nestlings' wastes was significantly different during the early and later day–ages. Moreover, the differences in the

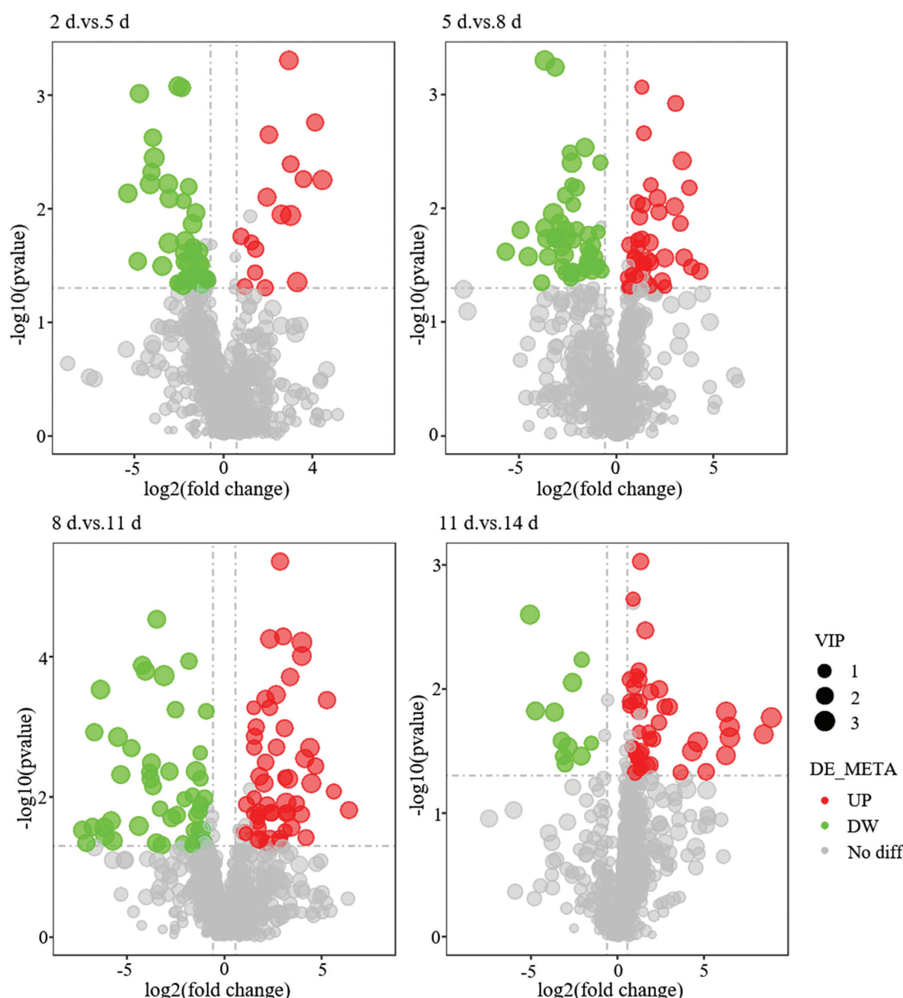

Figure 5. Analysis of comparison group of the gray-backed shrike nestlings' feces (2 vs. 5, 5 vs. 8, 8 vs. 11, and 11 vs. 14 day–ages) of the volcanic map. The abscissa represents the difference of metabolites in different groups (log2foldchange). The ordinate represents the different significance levels (−log10 *P*-value). Each point of the map represents a metabolite. The significantly upregulated metabolites are represented by red points, the significantly downregulated metabolites are represented by green points. The size of the dots represents the Variable Importance in the Projection value. FC < 0, metabolites were downregulated; FC > 0, metabolites were downregulated. The number of upregulated and downregulated metabolites in the 11 and 14 day–ages group was less than that in other control groups.

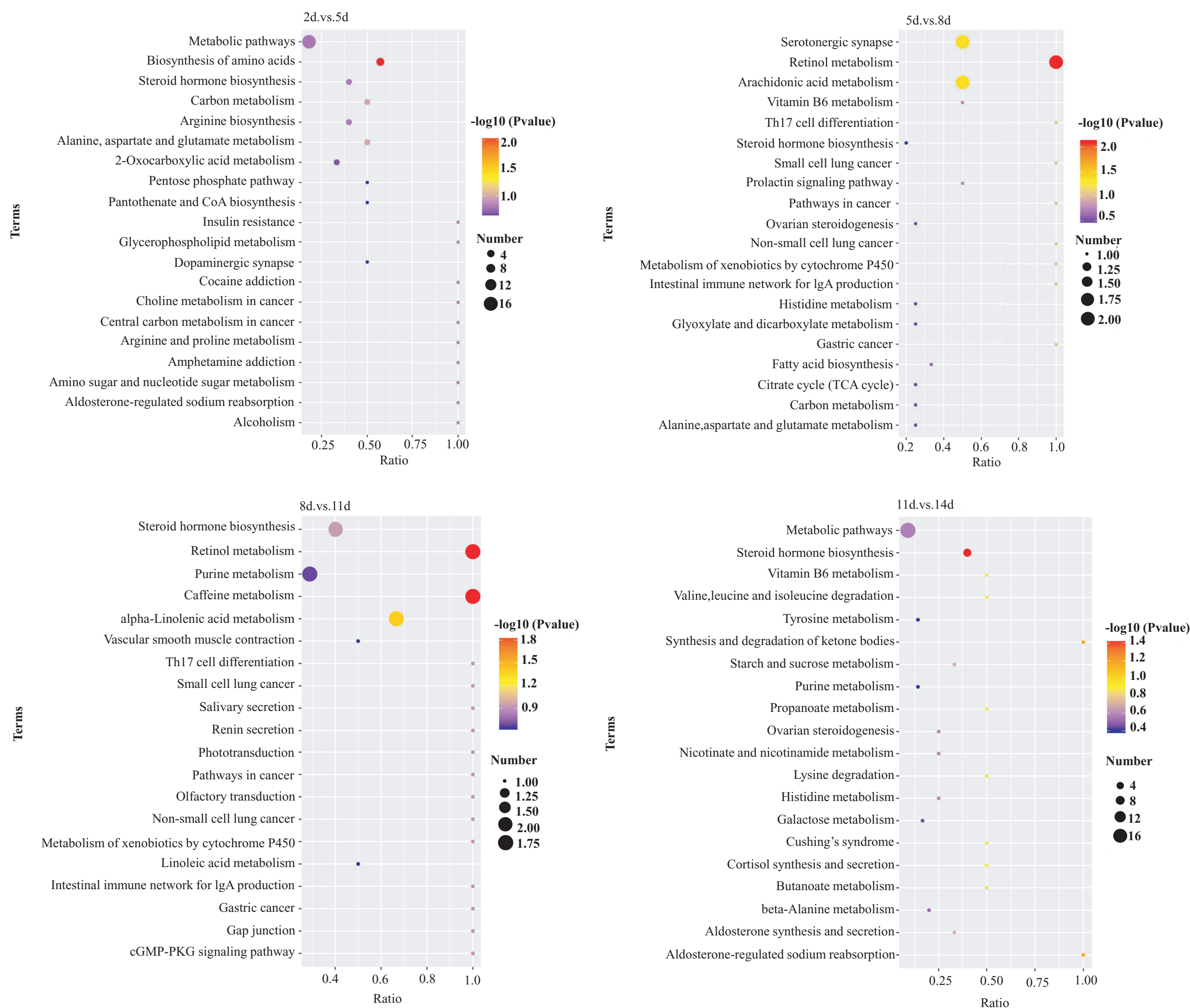

Figure 6. The KEGG enrichment map of fecal metabolites of nestlings in different contrast groups (2 vs. 5, 5 vs. 8, 8 vs. 11, and 11 vs. 14 day–ages), the smaller the *P*-value. The differentially expressed metabolites of the gray-backed shrike nestlings' feces comparison group (2 vs. 5, 5 vs. 8, 8 vs. 11, and 11 vs. 14 day–ages) are different.

community structure among individuals of the same day–age were very significant (Figure 1B,C). Our results are similar to a study that paid particular attention to microbiota changes of wild house sparrow nestlings *Passer domesticus* (Kohl et al. 2019). The gut microorganisms of nestlings show significant changes because of the microorganisms undergo a process of colonization (Benson et al. 2010; Tannock 2007; Turnbaugh et al. 2009), and the change in composition and structure of microorganisms is closely related to the environment of nestlings. After hatching, the nestlings are immediately affected by various factors because of the surrounding environment, and the microorganisms in the environment can develop rapidly in the intestinal tract of the nestlings within a short time (Pan and Yu 2014; Ding et al. 2017), which was probably similar to that of infants (Palmer et al. 2007). Nestlings develop quickly before they fly out. We believe that the improvement of the nestlings' immune system and digestive system also plays a crucial role in the growth of microorganisms in the intestine; this may explain the α-diversity of a 5-day-old nestling being lesser than that of a 2-day-old nestling (Table 1). The randomness of obtaining food may lead to significant differences in the microbial community structure among individuals of the same age (Table 1 and Figure 1;Sonnenburg et al. 2010; Hooper et al. 2012).

Regular fluctuation of the microbiota composition with the age of the nestlings was not observed at the phylum and genus levels, but the results showed that there was a notable change in the abundance of dominant fecal microorganisms of the nestlings when they are 8 days old (Figure 2A B). The fluctuation of these dominant bacteria may be related to the environment (Santacruz et al. 2009, 2010; Drewnowski and Rolls 2012; Hird et al. 2015) and food or the development of nestlings, such as weight gain (Santacruz et al. 2009, 2010; Drewnowski and Rolls, 2012) and the improvement of the intestinal immune system (Yang et al. 2018). It has been observed that the parental behavior with respect to the mode of feces disposal generally changes ~1 week after the nestlings hatch. Therefore, the change in the parental behavior with respect to feces disposal may be related to the change in the intestinal microbial composition of the 8-day-old nestlings. We used video (11,040 min), in the whole stage, the types of food provided by the parent bird to the nestlings during the whole stage did not change, there was no

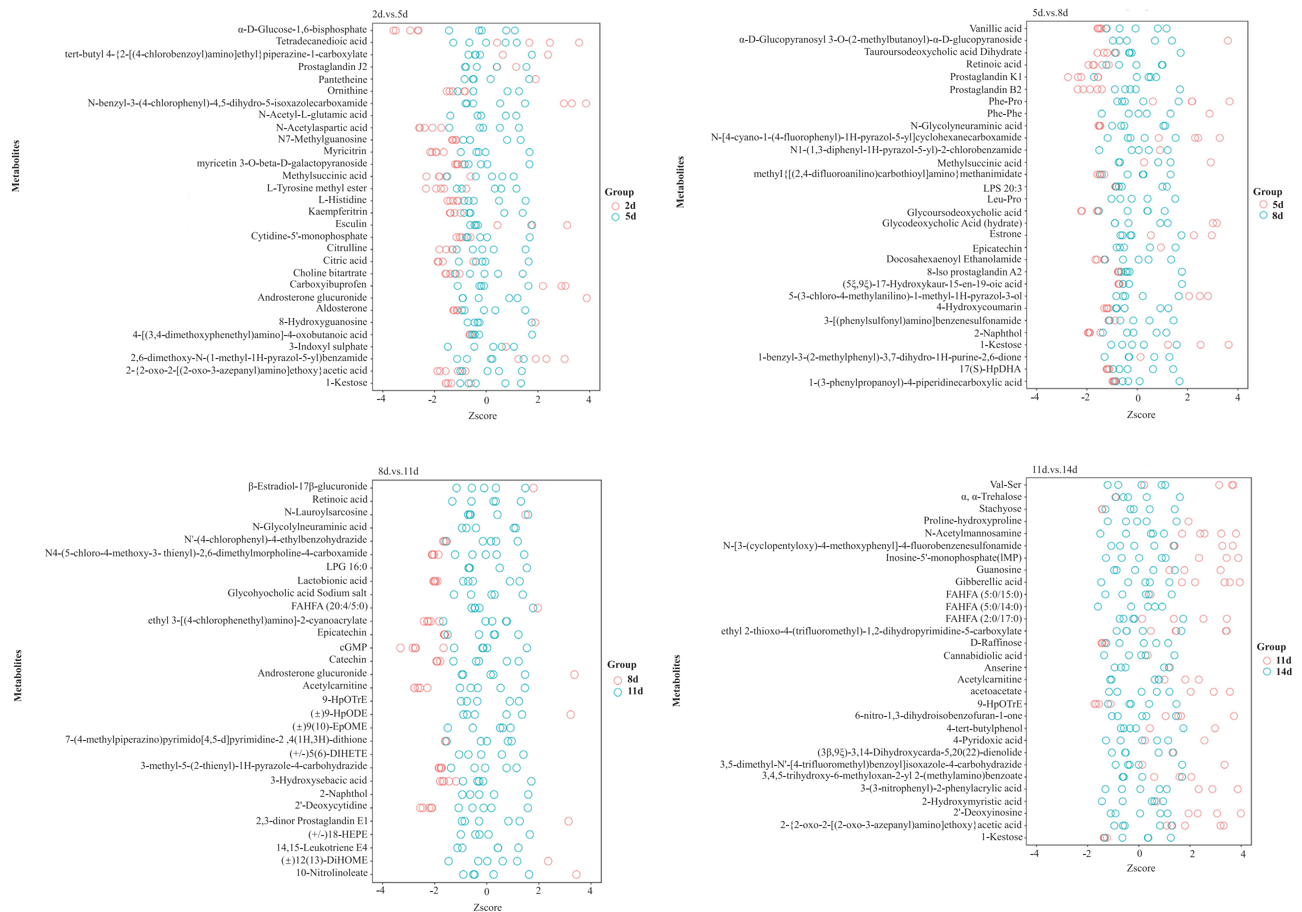

Figure 7. Analysis of *Z*-score, the abscissa is the *Z*-score value, the ordinate is the metabolite with a difference between the 2 control groups (2 vs. 5, 5 vs. 8, 8 vs. 11, and 11 vs. 14 day–ages), and each circle represents a sample. The middle value of the graph shows the top 30 metabolites sorted by *P*-value from small to large, and the *Z*-score values exceeding 4 or −4 has not been shown in the graph. When the fecal metabolite compositions of 8- and 11-day-old nestlings were compared, maximum change was observed between the numbers of differentially expressed metabolites.

significant correlation between nestlings day–age and food type ($r = 0.04$, $P > 0.05$; Supplementary Figure S3 and Supplementary Material S1), so the effect of food changes on fecal composition was minute. Furthermore, we analyzed the changes in the relative abundances of all the intestinal microorganisms with the increasing age of the nestlings, and it was observed that the relative abundances of 7 microorganisms gradually decreased, while that of 2 microorganisms gradually increased with the growth of the nestlings (Figure 3). In particular, among the 7 microorganisms with reduced abundance, except for Rombotusia whose function has not been confirmed (Gerritsen et al. 2019), the other 6 were probiotics. Most common probiotics can produce short-chain fatty acids (Afrc, 1989; Ma et al. 2020); they have the ability to adhere to intestinal cells, inhibit the binding of pathogens through competitive rejection process, and may affect the synthesis of cytokines. Moreover, the metabolites produced by these bacteria interact with other microorganisms to balance the intestinal microbiota as well as to improve the intestinal digestive activity. Probiotics help prevent malnutrition, promote protein synthesis (Sagheddu et al. 2016), and positively affect hosts' health by their active immunoregulation function (Caslin et al.2019; Supplementary Table S1 and Supplementary Material S1). Due to their beneficial effects on their hosts, they have been added to animal and human diets as common nutrients and dietary supplements, and this has made a positive impact on animal and human health (Kailasapathy and Chin, 2000; Qaisrani et al. 2015; Whitman et al. 2015). These probiotics varied with the age of nestlings, and their abundance decreased significantly after 8 days of age, which was consistent with our prediction. We believe that if these probiotics entered and colonized the parent birds' intestines through food, then there may be improvements in the parent birds' intestinal balance, a positive impact on their digestion and absorption of food, and an enhancement of the parent birds' intestinal immunity. Probiotics can maximize the nutrition of parent birds, compensate for the energy expended during brooding (to a certain extent), and play a beneficial role in their health. In conclusion, obtaining probiotics from the nestlings' feces may be the reason for the parental behavior of fecal matter ingestion. However, the content of probiotics in the nestlings' feces was low; hence, the influence of probiotics on the mode of disposal of nestlings' feces by the parent birds needs to be further studied on the basis of the specific content of probiotics and their ecological significance.

We identified 734 metabolites from all the fecal samples. The results showed that the differences in the composition of fecal metabolites gradually decreased with the age of the nestlings (Figures 4 and 5). The fecal metabolites of 2-, 5-, and 8-day-old nestlings were compared with those of 11- and 14-day-old nestlings (Table 2). Five metabolites were significantly upregulated in each comparison pair, among which 9-KODE, LPI 16:1, and LPI

Table 2 Difference in metabolites between the fecal ingestion stage and the fecal discarding stage: comparison of the metabolites in the feces of gray-backed shrike nestlings between fecal intake stage (2, 5, and 8 day–ages) and fecal discarding stage (11 and 14 day–ages)

Compared groups	2 days vs. 11 days			2 days vs. 14 days			5 days vs. 11 days			5 days vs. 14 days			8 days vs. 11 days			8 days vs. 14 days		
Compound name	log2FC	*P*-value	VIP	log2FC	*P*-value	VIP	log2FC	*P*-value	VIP	log2FC	*P*-value	VIP	log2FC	*P*-value	VIP	log2FC	*P*-value	VIP
Up																		
9-KODE	2.6872	0.04560	1.2253	3.4171	0.01521	1.6301	1.9260	0.00961	1.4776	2.6568	0.00332	1.8715	2.8490	0.03899	1.6075	3.5789	0.01333	1.8980
CUMYL-PICA N-pentanoic acid metabolite	1.7004	0.01397	1.1968	2.6788	0.00671	1.6078	2.9657	0.00151	2.1222	3.1446	0.00036	2.2441	2.3975	0.01700	1.5916	2.5764	0.00782	1.6421
LPI 16:1	4.2996	0.04091	1.2290	5.9408	0.01461	1.7316	3.8588	0.01990	1.4573	5.5001	0.00607	1.9296	3.1235	0.00531	1.3928	4.7648	0.00065	1.8007
LPI 18:2	5.8062	0.02709	1.1522	8.1732	0.00492	1.6210	4.8727	0.00455	1.6762	7.2396	0.00021	2.1064	4.6848	0.00363	1.7189	7.0517	5.96E-05	2.0690
Xanthosine	3.1147	0.00837	1.7583	2.6089	0.01580	1.5377	1.9394	0.01281	1.4502	1.4336	0.03316	1.1298	2.2053	0.01294	1.6021	1.6995	0.03117	1.1943
Down																		
3-(pyrazin-2-ylamino)-2-(2-thienylcarbonyl)acrylonitrile	−4.8702	0.00246	1.9776	−4.7747	8.11E-05	2.1732	−2.4680	0.03809	1.4985	−2.3725	0.01602	1.6079	−3.7827	0.00554	1.9207	−3.6872	0.00053	1.9284
3-methyl-5-(2-thienyl)-1H-pyrazole-4-carbohydrazide	−3.4297	0.00053	1.5625	−3.2376	0.01293	1.3274	−3.3530	0.00049	1.9306	−3.1610	0.01163	1.5471	−4.0489	0.00016	2.2855	−3.8568	0.00502	1.8006
7-(4-methylpiperazino)pyrimido[4,5-d]pyrimidine-2,4(1H, 3H)-dithione	−7.9637	0.00036	1.9247	−6.8182	0.00067	1.7192	−4.3363	0.01381	1.7085	−3.1908	0.02889	1.3670	−6.6692	0.00119	1.9369	−5.5236	0.00232	1.5184
Catechin	−3.9741	0.00125	1.3825	−3.1557	0.01118	1.0303	−4.7985	0.00020	1.9589	−3.9801	0.00282	1.4476	−6.3552	0.00029	2.3567	−4.6433	0.00314	1.8079
Dibenzoyl Thiamine	−2.8877	0.01128	1.5511	−2.2765	0.01457	1.3339	−3.7167	0.00489	2.1084	−3.1055	0.00238	1.7453	−3.7418	0.00327	2.1991	−3.1306	0.00178	1.7608
Estrone sulfate	−3.9083	0.00831	1.5030	−4.1508	0.00557	1.6859	−4.3816	0.00604	2.0846	−4.624	0.00429	2.1665	−3.6630	0.00705	1.7838	−3.9055	0.00427	1.8137
ethyl 3-[(4-chlorophenethyl)amino]-2-cyanoacrylate	−2.3808	0.00071	1.3048	−2.5136	0.00950	1.3051	−2.2892	0.00227	1.4766	−2.4220	0.01657	1.3836	−2.5058	0.00057	1.6985	−2.6386	0.00694	1.5298
Jasmonic acid	−5.2351	0.00706	1.6810	−5.4463	0.00022	1.9515	−5.4315	0.00638	2.1444	−5.6427	0.00024	2.3172	−5.3121	0.00480	2.1587	−5.5233	9.28E-05	2.2493
Lactobionic acid	−3.9061	1.02E-05	1.9523	−2.8803	0.00048	1.4242	−3.8073	8.98E-05	2.2952	−2.7815	0.00148	1.5499	−3.4662	2.91E-05	2.1075	−2.4404	0.00143	1.3168
N1-(4-chlorophenyl)-2-cyano-4,4-dimethyl-3-oxopentanamide	−6.8677	0.00049	1.7099	−6.7365	0.00667	1.5551	−4.2014	0.00307	1.6619	−4.0701	0.01872	1.3919	−3.8336	0.00441	1.5147	−3.7024	0.02884	1.1896
Phloretin	−4.8114	0.00441	1.4387	−5.3988	0.00974	1.4914	−2.9717	0.02349	1.3302	−3.5591	0.03514	1.3098	−3.2925	0.01474	1.4143	−3.8780	0.02470	1.3337

VIP, Variable Importance in the Projection.

18:2 were lipids (Supplementary Table S2 and Supplementary Material S1); on the contrary, 11 metabolites were significantly downregulated, and lipids were absent. The composition of metabolites in feces varies when nestlings are early day–ages, but it is similar when they are late (Figure 4), which may be related to the gradual maturation of their digestive systems. Lipids are easily metabolized and absorbed as a part of the diet (Osborn-Barnes and Akoh 2005); thus, the nutritional value of feces would be insufficient when their contents decreased. Besides, the downregulated metabolites may be harmful to birds health, such as catechin, which is a bitter compound that can reduce intestinal lipid metabolism (Ikeda et al. 1992; Higdon and Frei 2003) (Supplementary Table S2 and Supplementary Material S1). Along the time period, it was found that the difference of nestling metabolites was the largest between 8th and 11th day after hatching (Figure 7). Additionally, we performed a statistical analysis of the changing trends of all the identified metabolites and found that 60 of them decreased with the growth of nestlings, among which 20 are lipids, which may have provided energy to the parent birds when they were present in abundance in the nestlings' feces. On the contrary, with the increase in the age of the nestlings, the content of 42 metabolites significantly increased, among which only 5 were lipids, and most of the non-lipid metabolites were neutral compounds. In the high-frequency time, when the parent birds' fecal treatment strategy changed, the metabolites in the feces changed the most when the nestlings were $\sim$ 8 days old. These results support our hypothesis regarding influence of fecal composition on the parent birds' fecal eating behavior.

Meanwhile, from reducing lipid metabolites in feces, we can intuitively predict that the nutrient composition of the wastes of older nestlings was poor. Among the nonlipid metabolites which decreased as the nestlings grew old, some have had close links with birds' health. The beneficial metabolites can protect them against diseases (Peeters and Halen 1980) and be useful for wound healing and relieving skin irritation (Goelzer et al. 2008; Supplementary Table S2 and Supplementary Material S1). During the brooding period, the antibiotic effect of beneficial metabolites helps them reduce the health risk caused by parasites, so as to improve their survival rate. We believe that parent birds demand metabolites that are good for skin healing because their nests are usually built on thorny plants; they may be scratched when passing through these thorns. In short, the observation that younger the nestlings have more lipids and beneficial substances in their feces compared with the older nestlings, and the fact that these beneficial substances decrease over time, is consistent with our conjecture.

Therefore, the parent birds may ingest the feces as an additional source of probiotics and beneficial metabolites; our results are in accordance with the "parental nutrition hypotheses." However, owing to the limited number of gray-backed shrike nests found every year, the fields' sampling operation faces many unpredictable challenges, such as nest predation or extreme weather. We must admit that the sample size collected is minimal, and these samples can be used for statistically significant experiments. However, if more samples can be collected in some species with a large population, more universal conclusions may be drawn.

Ethical Statement

All experimental protocols were approved by the Institute of Animal Ecology, Lanzhou University.

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Conflict of Interest Statement

There are no conflicts of interest to declare.

Supplementary Material

Supplementary material can be found at https://academic.oup.com/cz.

References

Afrc RF, 1989. Probiotics in man and animals. *J Appl Bacteriol* **66**:365–378.

Alberts JR, Gubernick DJ, 1983. Reciprocity and resource exchange. In: Rosenblum LA, Moltz H editors. *Symbiosis in Parent-Offspring Interactions*. Boston (MA): Springer, 7–44.

Azcárate-García M, Ruiz-Rodríguez M, Díaz-Lora S, Ruiz-Castellano C, Soler JJ, 2019. Experimentally broken faecal sacs affect nest bacterial environment, development and survival of spotless starling nestlings. *J Avian Biol* **50**:1–10.

Aziz Q, Doré J, Emmanuel A, Guarner F, Quigley EMM, 2013. Gut microbiota and gastrointestinal health: current concepts and future directions. *Neurogastroenterol Motil* **25**:4–15.

Barri T, Dragsted LO, 2013. UPLC-ESI-QTOF/MS and multivariate data analysis for blood plasma and serum metabolomics: Effect of experimental artefacts and anticoagulant. *Anal Chim Acta* **768**:118–128.

Benson AK, Kelly SA, Legge R, Ma FR, Low SJ et al., 2010. Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors. *Proc Natl Acad Sci U S A* **107**: 18933–18938.

Bokulich NA, Subramanian S, Faith JJ, Gevers D, Gordon JI et al., 2013. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nat Methods* **10**:57–59.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD et al., 2010. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* **7**:335–336.

Caslin B, Maguire C, Karmakar A, Mohler K, Wylie D et al., 2019. Alcohol shifts gut microbial networks and ameliorates a murine model of neuroinflammation in a sex-specific pattern. *Proc Natl Acad Sci U S A* **116**: 25808–25815.

Dell'omo G, Alleva E, Carere C, 1998. Parental recycling of nestling faeces in the common swift. *Anim Behav* **56**:631–637.

Ding J, Dai R, Yang L, He C, Xu K et al., 2017. Inheritance and establishment of gut microbiota in chickens. *Front Microbiol* **8**:1967.

Drewnowski A, Rolls B, 2012. Concluding remarks. In: Drewnowski A, Rolls B, editors. *Obesity Treatment and Prevention: New Directions*. Basel, Switzerland: S. Karger AG, 143–145.

Edgar RC, 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* **32**:1792–1797.

Edgar RC, 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat Methods* **10**:996–998.

Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R, 2011. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **27**: 2194–2200.

Gerritsen J, Hornung B, Ritari J, Paulin L, Rijkers GT et al., 2019. A comparative and functional genomics analysis of the genus *Romboutsia* provides insight into adaptation to an intestinal lifestyle. *bioRxiv* 845511. doi: https://doi.org/10.1101/845511.

Glück E, 1988. Why do parent birds swallow the feces of their nestlings? *Experientia* **44**:537–539.

Goelzer A, Bekkal Brikci F, Martin-Verstraete I, Noirot P, Bessières P et al., 2008. Reconstruction and analysis of the genetic and metabolic regulatory networks of the central metabolism of Bacillus subtilis. *BMC Syst Biol* **2**:20.

Haas BJ, Gevers D, Earl AM, Feldgarden M, Ward DV et al., 2011. Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. *Genome Res* **21**:494–504.

Higdon JV, Frei B, 2003. Tea catechins and polyphenols: Health effects, metabolism, and antioxidant functions. *Crit Rev Food Sci Nutr* **43**:89–143.

Hird SM, Sánchez C, Carstens BC, Brumfield RT, 2015. Comparative gut microbiota of 59 neotropical bird species. *Front Microbiol* **6**:1403.

Hooper LV, Littman DR, Macpherson AJ, 2012. Interactions between the microbiota and the immune system. *Science* **336**:1268–1273.

Hurd PL, Weatherhead PJ, McRae SB, 1991. Parental consumption of nestling feces: good food or sound economics? *Behav Ecol* **2**:69–76.

Ibáñez-Álamo JD, Ruiz-Rodríguez M, Soler JJ, 2014. The mucous covering of fecal sacs prevents birds from infection with enteric bacteria. *J Avian Biol* **45**:354–358.

Ikeda I, Imasato Y, Sasaki E, Nakayama M, Nagao H et al., 1992. Tea catechins decrease micellar solubility and intestinal absorption of cholesterol in rats. *Biochim Biophys Acta* **1127**:141–146.

Kailasapathy K, Chin J, 2000. Survival and therapeutic potential of probiotic organisms with reference to *Lactobacillus acidophilus* and *Bifidobacterium* spp. *Immunol Cell Biol* **78**:80–88.

Kohl KD, Brun A, Caviedes-Vidal E, Karasov WH, 2019. Age-related changes in the gut microbiota of wild house sparrow nestlings. *Ibis* **161**:184–191.

Lang JD, Straight CA, Gowaty PA, 2002. Observations of fecal sac disposal by eastern bluebirds. *Condor* **104**:205–207.

Lima SL, 1986. Predation risk and unpredictable feeding conditions: Determinants of body mass in birds. *Ecology* **67**:377–385.

Lindsay EC, Metcalfe NB, Llewellyn MS, 2020. The potential role of the gut microbiota in shaping host energetics and metabolic rate. *J Anim Ecol* **89**:2415–2426.

Lu X, Wang C, Yu TL, 2010. Nesting ecology of the grey-backed shrike *Lanius tephronotus* in south Tibet. *Wilson J Ornithol* **122**:395–398.

Ma QT, Li YQ, Wang JK, Li PF, Duan YH et al., 2020. Investigation of gut microbiome changes in type 1 diabetic mellitus rats based on high-throughput sequencing. *Biomed Pharmacother* **124**:109873.

Magoč T, Salzberg SL, 2011. FLASH: Fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* **27**:2957–2963.

Morton ML, 1979. Fecal sac ingestion in the mountain white-crowned sparrow. *Condor* **81**:72.

Morton ML, Orejuela JE, Budd SM, 1972. The biology of immature mountain white-crowned sparrows *Zonotrichia leucophrys oriantha* on the breeding ground. *Condor* **74**:423–430.

Negro JJ, Grande JM, Tella JL, Garrido J, Hornero D et al., 2002. An unusual source of essential carotenoids. *Nature* **416**:807–808.

Osborn-Barnes HT, Akoh CC, 2005. Application of structured lipids in emulsions. In: Akoh CC editor. *Handbook of Functional Lipids*. Boca Raton (FL): CRC Press, 457–473.

Palmer C, Bik EM, DiGiulio DB, Relman DA, Brown PO, 2007. Development of the human infant intestinal microbiota. *PLoS Biol* **5**:e177.

Pan D, Yu ZT, 2014. Intestinal microbiome of poultry and its interaction with host and diet. *Gut Microbes* **5**:108–119.

Pappas S, Benson TJ, Bednarz JC, 2010. Effects of brown-headed cowbird parasitism on provisioning rates of swainson's warblers. *Wilson J Ornithol* **122**:75–81.

Peeters JE, Halen P, 1980. Robenidine treatment of rabbits naturally infected with coccidia. *Lab Anim* **14**:53–54.

Petit KE, Petit LJ, Petit DR, 1989. Fecal sac removal: Do the pattern and distance of dispersal affect the chance of nest predation? *Condor* **91**:479.

Qaisrani SN, van Krimpen MM, Kwakkel RP, Verstegen MWA, Hendriks WH, 2015. Diet structure, butyric acid, and fermentable carbohydrates influence growth performance, gut morphology, and cecal fermentation characteristics in broilers. *Poult Sci* **94**:2152–2164.

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T et al., 2013. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Res* **41**:D590–D596.

Sagheddu V, Patrone V, Miragoli F, Puglisi E, Morelli L, 2016. Infant early gut colonization by lachnospiraceae: High frequency of ruminococcus gnavus. *Front Pediatr* **4**:57.

Santacruz A, Collado MC, García-Valdés L, Segura MT, Martín-Lagos JA et al., 2010. Gut microbiota composition is associated with body weight, weight gain and biochemical parameters in pregnant women. *Br J Nutr* **104**:83–92.

Santacruz A, Marcos A, Wärnberg J, Martí A, Martin-Matillas M et al., 2009. Interplay between weight loss and gut microbiota composition in overweight adolescents. *Obesity* **17**:1906–1915.

Skadhauge E, 1981. A brief survey of hormones and osmoregulation. In: Skadhauge E editor. *Osmoregulation in Birds*. Berlin, Germany: Springer Berlin Heidelberg, 151–155.

Sonnenburg ED, Zheng HJ, Joglekar P, Higginbottom SK, Firbank SJ et al., 2010. Specificity of polysaccharide use in intestinal bacteroides species determines diet-induced microbiota alterations. *Cell* **141**:1241–1252.

Speakman JR, Chi Q, Ołdakowski Ł, Fu H, Fletcher QE et al., 2021. Surviving winter on the Qinghai-Tibetan Plateau: Pikas suppress energy demands and exploit yak feces to survive winter. *Proc Natl Acad Sci U S A* **118**:e2100707118.

Spencer KA, 2005. The effects of body state on nest sanitation and provisioning effort in breeding barn swallows *Hirundo rustica*. *Can J Zool* **83**:1360–1364.

Tannock GW, 2007. What immunologists should know about bacterial communities of the human bowel. *Semin Immunol* **19**:94–105.

Thomson DF, 1934. Some adaptations for the disposal of fæces: The hygiene of the nest in australian birds. *Proc Zool Soc London* **104**:701–708.

Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A et al., 2009. A core gut microbiome in obese and lean twins. *Nature* **457**:480–484.

Want EJ, O'Maille G, Smith CA, Brandon TR, Uritboonthai W et al., 2006. Solvent-dependent metabolite distribution, clustering, and protein extraction for serum profiling with mass spectrometry. *Anal Chem* **78**:743–752.

Wen B, Mei ZL, Zeng CW, Liu SQ, 2017. metaX: A flexible and comprehensive software for processing metabolomics data. *BMCBioinformatics* **18**:183.

Whitman WB, Chun J, Dedysh S, De Vos P, Hedlund BP, et al., 2015. *Bergey's Manual of Systematics of Archaea and Bacteria*. New York (NY): John Wiley & Sons, Inc, Bergey's Manual Trust.

Witherby HF, 1924. A practical handbook of British birds. *Nature* **114**:119.

Yang LY, Liu C, Zhao WJ, He C, Ding JM et al., 2018. Impaired autophagy in intestinal epithelial cells alters gut microbiota and host immune responses. *Appl Environ Microbiol* **84**:e00880–e00818.

Zack S, 1986. Breeding biology and inter-territory movements in a fiscal shrike population in Kenya. *Ostrich* **57**:65–74.