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Research Article

Age-related Changes in the Cloacal Microbiota of Bar-headed Geese *(Anser indicus)*

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Abstract

The gastrointestinal microbiota played an important role in animal health by acting as a barrier against pathogens, exerting multiple metabolic functions and stimulating the development of the host immune system. To better understand the age-related dynamic changes in gut microbiota, we used 16S rRNA genes sequencing to investigate the cloacal microbial communities of the adult and chick bar-headed geese *(Anser indicus). Fusobacteria, Firmicutes, Proteobacteria, Actinobacteria* were the main components shared by adults and chicks. The former had more *Proteobacteria* and *Cyanobacteria* and the latter had more *Fusobacteria* and *Actinobacteria*. At the genus level, most of the dominant genera found in chicks were different from those in adults. In addition, adults had richer and more diverse bacterial communities than chicks. Our analysis of the composition of cloacal microbiota at the OTUs level also showed very large overlap existed in the bacterial assemblages between chicks and adults. These overlapped microbes were considered as the major microbes in the gastrointestinal tracts of bar-headed geese throughout their whole life span. Taken together, the results of this study provided a first inventory of the gut microbiotas of chick bar-headed geese and represented a first step in a wider investigation of the sequential changes in gut microbiotas with ages in bar-headed geese.

Keywords: *Bar-headed goose, Cloacal microbial community, 16S rRNA genes*

Hint Kazı *(Anser indicus)*'nın Kloaka Mikrobiyotasında Yaşa Bağlı Değişiklikler

Öz

Gastrointestinal mikrobiyota, patojenlere karşı bir engel görevi görerek, çoklu metabolik fonksiyonları yerine getirerek ve konakçı bağışıklık sisteminin gelişimini uyararak hayvan sağlığında önemli bir rol oynar. Bağırsak mikrobiyotasında yaşa bağlı dinamik değişimleri daha iyi anlayabilmek için, yetişkin ve civciv Hint kazlarının *(Anser indicus)* kloakal mikrobiyal yapısını araştırmak amacıyla 16S rRNA gen sekanslaması kullanıldı. F*usobacteria, Firmicutes, Proteobacteria* ve *Actinobacteria* yetişkin ve civciv kazlar tarafından ortak paylaşılan bileşenlerdi. Yetişkinlerde *Proteobacteria* ve *Cyanobacteria* civcivlerde *Fusobacteria* ve *Actinobacteria* daha fazlaydı. Civcivlerde bulunan baskın genusun çoğu yetişkinlerinkinden farklıydı. Ayrıca, yetişkinler civcivlerden daha zengin ve daha farklı bakteriyel mikrobiyotaya sahipti. OTU seviyesinde kloakal mikrobiyotanın analizi, civcivler ve yetişkinler arasında bakteriyel topluluklar bakımından büyük ölçüde örtüşmenin olduğunu gösterdi. Bu örtüşen mikroorganizmaların, Hint kazlarının hayatları süresince gastrointestinal kanallarının ana mikroorganizmaları olduğu düşünülmektedir. Çalışma, Hint kazlarının bağırsak mikrobiyotasını belirlemiş ve yaş ile bağırsak mikrobiyotasında gelişen değişiklikleri belirlemek amacıyla yapılacak detaylı çalışmalar için bir adım atmıştır.

Anahtar sözcükler: *Hint kazı, Kloakal Mikrobiyota Bileşenleri, 16S rRNA geni*

INTRODUCTION

In animals, microorganisms occur both externally (e.g. skin and feather) and internally (e.g. gastrointestinal and reproductive tracts) of their hosts [1]. The majority of the microorganisms associated with animals inhabit the gastrointestinal tracts at an abundance of potentially trillions of cells whose collective genome named "gut microbiome" [2]. A wealth of studies have shown that gut microbiome plays an important role in several fundamental and crucial

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processes such as development [3], immune homeostasis [4], nutrient assimilation [5,6], vitamins synthesis and sterols metabolism in the host [7], and diseases (e.g., obesity, diabetes, and cancer) in humans and other animals [8].

Birds are endothermic, feathered amniotes with 10.659 described species and more than 20.413 subspecies [9]. Compared to other mammalian vertebrates, several characteristics make birds the most interesting and useful model for studying gut microbiome. First, unlike other mammals where host genetics have shown a clear influence on the colonizing process of gut microbiota [10], birds are more likely to acquire microbes after hatching from the nest environments or food. For example, birds brood parasites offer a unique and powerful model to investigate these questions because genetic and environmental transmission of microbes are naturally decoupled. A study of Brown-headed cowbirds *(Molothrus ater)*, a brood parasite that relies on other species to hatch and raise their young, found that gut microbiome was not related to host species, but rather to environments [11]. Secondly, many birds regurgitate food to their young, thus provides a mode of vertical transmission of gut microbiome across generations, whereas mammals acquire important maternal microbes during the birth process [12]. Thirdly, birds possess a cloaca, which serves the dual functions of excretion and sexual copulation. Thus, gastrointestinal tract microbiotas of birds provide another avenue for exploring the potential exchange of components of the endogenous microbiome during reproduction. For example, Kreisinger et al.[13] described the cloacal microbiomes in free living barn swallows *(Hirundo rustica)* and found that nesting pairs had significantly more similar microbiomes within pairs than between nonbreeding individuals. Lastly, in the fieldwork, we have noticed that some bird species lived in mouse holes. This phenomenon, birds and mouse share the same living environments, provides another opportunity to understand the coevolution of the gut microbiotas with different hosts (birds and mammals) in the same environments.

In general, compared to other mammalian vertebrates, we know much less about the gut microbiota of wild birds [14]. The majority of avian microbiome studies have focused on economically important species such as chicken [15], turkey, duck and ostrich [16]. The reasons for this are various but may relate to the collection of biological samples (especially feces) from several groups of wild birds is a difficult task. Similar to other vertebrates, the gut microbiota of birds are dominated by the four major phyla [17]: *Firmicutes*, *Proteobacteria*, *Actinobacteria* and *Bacteroidetes*.

The bar-headed goose *(Anser indicus)* is endemic to Asia, breeding in the Mongolia plateau of central Asia and the Qinghai-Tibet plateau in China, wintering in the south-central Tibet and South-Asian subcontinent [18]. As one of the dominant waterfowl species in wetland areas in Qinghai-Tibetan Plateau, bar-headed geese are increasingly being reared in several provinces of China since year 2003 for the purpose of both conservation and economic development [19]. In the early stage of industrialization of this bird, a limited number of wild eggs were collected and then artificially incubated using an incubator. The gastrointestinal tracts of newly-hatched chicks are immediately colonized by microorganisms present in the surrounding artificial environments. By contrast, in the wild, the gastrointestinal tracts of newly-hatched chicks are rapidly colonized by members of the gut microbiomes from its mother's feces and nest environments. In many bird species, the chicks' gut microbiotas are dynamical changing communities that gradually develops toward the adult community structure [20]. Therefore, figuring out the gut microbiomes of wild chick bar-headed geese is important for the management of the artificially reared chicks. In our previous studies [21], we have found that the core gut microbiomes of wild bar-headed geese were dominated by *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes*. Furthermore, *Bacteroidetes* were found to be higher in artificially reared bar-headed geese compared to wild ones [22]. However, it remains unclear how the normal gut microbiome changes between young and adult wild bar-headed geese.

Here, we describe for the first time the cloacal microbiota in bar-headed geese comparing both adults and chicks and to analyze the similarities and differences between them. Cloacal swabs were selected for high-throughput sequencing of 16S rRNA V3-V4 regions in this study, because it was not feasible to obtain naturally passed feces from wild chicks. Cloacal swabs are believed to at least partially reflect the microbiota present in the gastrointestinal tracts [23] and do not require invasive sampling. Our results form an important basis for understanding changes in gut microbiota compositions and patterns with host age in wild birds.

MATERIAL and METHODS

Ethics Statement

This study conformed to the guidelines for the care and use of experimental animals established by the Ministry of Science and Technology of the People's Republic of China (Approval number: 2006-398). The research protocol was reviewed and approved by the Ethical Committee of Qinghai University. Samples collection was authorized by the officer Yubang He from the Administration Bureau of Qinghai Lake National Nature Reserve, Qinghai Province, China. All wild bar-headed geese were released at the capture site immediately after cloacal samples collection.

Samples Collection

A total of 5 wild bar-headed geese, 3 chicks (abbreviation: C group) and 2 adults (abbreviation: A group), were used in this study. These birds were captured using mist nets in

a farmland (N: 37° 01′ 39.3″, E: 99° 44′ 21.8″, Elevation: 3,200 m) adjacent to Bird Island of Qinghai Lake National Nature Reserve. Every day only one captured bird was randomly selected for cloacal sampling to minimize the overlap in bacterial assemblages between two individuals due to, for example, a shared nesting environments. Cloacal samples were collected using sterile DNA-free microbiological nylon swabs inserted about 10 mm inside the cloaca for approximate 20 s and gently twisted by 360 degrees. Swabs were placed into DNA-free sterile tubes and initially kept in car-refrigerator (-20°C), then shipped to the laboratory stored at -80°C until samples processing.

DNA Extraction, PCR Amplification, and Illumina HiSeq 2500 Sequencing

DNA extraction was performed on samples by using E.Z.N.A.® stool DNA Kit (Omega Bio-tek, Norcross, GA, USA) according to manufacturer's protocol. The purity and concentration were checked using NanoPhotometer (Implen, Westlake Village, CA USA) and Qubit2.0 Flurometer (Life Technologies, Carlsbad, CA, USA). The V3-V4 regions of the bacteria 16S rRNA genes were amplified by PCR (95°C for 3 min, followed by 25 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s, and a final extension at 72°C for 10 min) using primers 341F (5›-barcode-CCTACGGGNGGCWGCAG-3›) and 805R (5›-barcode-GACTACHVGGGTATCTAATCC-3›), where barcode is an eight-base sequence unique to each sample. PCR reactions were performed in triplicate, 20 µL mixture containing 4 µL of 5 X FastPfu buffer, 2 µL of 2.5 mM dNTPs, 0.8 µL of each primer (5 mM), 0.4 µL of FastPfu polymerase, and 10 ng of template DNA. PCR products were then run on 1% agarose gel and bands of appropriate size were extracted from the gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions using 30 µL of buffer in the elution step. Concentration of the purified PCR product was measured using a QuantiFluor™ - ST (Promega, Madison City, WI, USA). Purified amplicons were pooled in equimolar and paired - end sequenced (2 x 250) on an Illumina HiSeq2500 platform according to the standard protocol.

Data Accessibility

The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (accession number: SRP090120).

Data Analysis

The raw fastq files were demultiplexed based on the barcode and primer sequence with the following criteria: (i) exact barcode matching, (ii) 2 nucleotide mismatch in primer matching, (iii) reads containing ambiguous characters were removed. Then paired - end reads for each sample were run through Trimmomatic (version 0.33) [24] to remove low quality base pairs using these parameters [SLIDINGWINDOW: 50: 20 MINLEN: 50]. Trimmed reads were then further merged using FLASH program (version 1.2.8) [25] with the parameters [-m 10 -x 0.2 -p 33 -r 300 -f 450 -s 150].

The 16S sequences were analyzed using a combination of software UPARSE (usearch version v8.0.1517, http://drive5.com/uparse/) [26], QIIME (version 1.9.1) [27], and R (version 3.2.3). The cleaned reads were clustered at 97% sequence identity into operational taxonomic units (OTUs) using the UPARSE pipeline (http://drive5.com/usearch/manual/uparse_cmds.html). The OTU representative sequences were aligned against to the greengenes reference template set (http://greengenes.lbl.gov/Download/Sequence_Data/Fasta_data_files/core_set_aligned.fasta.imputed) based on PyNAST (version 1.2.2) [28]. The phylogenetic tree was constructed using FastTree (version 2.1.3) [29] with the filtered alignment. The Ribosomal Database Project (RDP) Classifier (version 2.2) [30] was employed for taxonomy assignment against Greengenes (version gg_13_8) [31] with confidence score > = 0.8.

For the alpha-diversity metrics, Chao1 and observed species were calculated by mothur (version 1.36) [32] and Rarefaction plots were generated with iterations of 10 at each sampling depth 3000 and increments of 100. Differences between two independent groups were evaluated by the Welch's t-test. P-values<0.05 were considered to be significant. All figures were generated with customized R scripts.

RESULTS

After alignment, gap removal, and potential chimera removal, nearly 512,818 valid clean reads were generated for cloacal bacteria, representing 256,388 assembled sequences with a median length of 450 bp from our dataset *(Table 1)*. These assembled sequences yielded a total of 916 distinct OTUs, ranged from 46 to 574, with a 97% sequence similarity threshold. *Table 2* showed the number of OTUs assigned to different taxonomic levels (from phylum to genus) in each sample.

A total of 9 different bacterial phyla were identified in the cloacal microbiotas of chicks *(Fig. 1A)*. The results showed that *Fusobacteria* predominated (48.29%) among chicks followed by *Firmicutes* (22.21%), *Proteobacteria* (22.07%), *Actinobacteria* (5.02%) and *Tenericutes* (1.93%) *(Table 3)*. A total of 17 different bacterial phyla were identified in the cloacal microbiotas of adults *(Fig. 1A)*. The top 5 most abundant phyla identified were: *Proteobacteria* (64.69%), *Firmicutes* (23.92%), *Cyanobacteria* (8.48%), *Actinobacteria* (1.43%) and *Fusobacteria* (0.56%) *(Table 3)*. Comparison at the phylum level showed that *Fusobacteria* (P=0.239) and *Actinobacteria* (P=0.125) abundances tended to increase in chicks, while *Proteobacteria* (P=0.211) and *Cyanobacteria* (P=0.136) abundances tended to increase in adults *(Table 3)*.

At the genus level, the sequences from the samples represented 18 and 24 genera in chicks and adults, respectively *(Fig. 1B)*. The sequences that could not be

Table 1. *Raw data before and after standard quality control filters*

Samples	Raw Reads	Raw Bases (bp)	Clean Reads	Clean Bases (bp)	Assembled Reads
C1	114.432	28.608.000	105.086	26.271.500	52.540
C2	114.860	28.715.000	103.966	25.991.500	51.978
C3	111.432	27.858.000	102.416	25.604.000	51.205
A1	112.320	28.080.000	101.044	25.261.000	50.517
A2	110.510	27.627.500	100.306	25.076.500	50.148

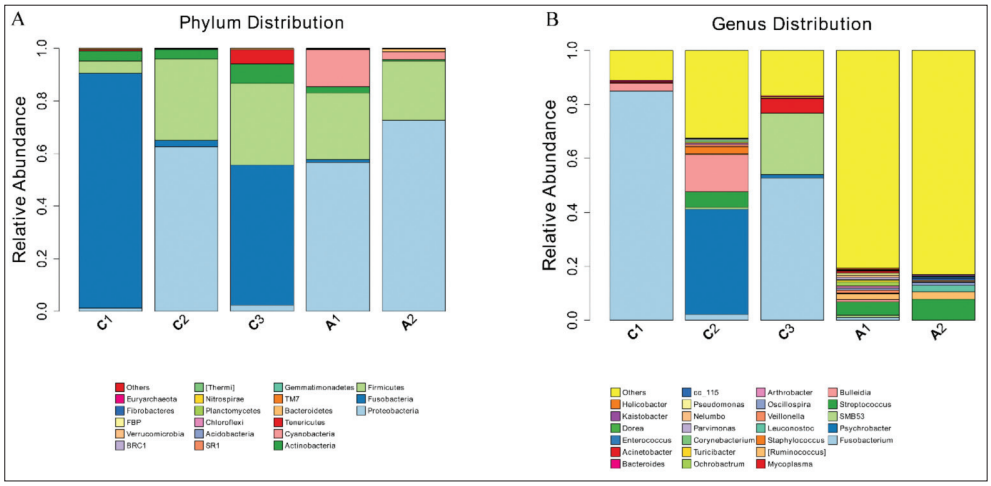

Fig 1. The phylum (A) and genus (B) level microbial compositions of each sample

Table 2. *The number of identified OTUs and taxonomic units in each sample*

Samples	OTUs	Number of Taxonomic Units				
		Phylum	Class	Order	Family	Genus
C1	46	7	13	14	13	11
C2	189	9	16	21	21	18
C3	133	7	15	21	21	15
A1	574	16	25	25	25	25
A2	509	18	24	24	24	23

Table 3. *Comparison of the top 5 most abundant phylum in each group*

Phylum	C Group	A Group	P Value
Fusobacteria	48.29%	0.56%	0.239
Firmicutes	22.21%	23.92%	0.890
Proteobacteria	22.07%	64.69%	0.211
Actinobacteria	5.02%	1.43%	0.125
Tenericutes	1.93%	-	0.454
Cyanobacteria	-	8.48%	0.136

classified into any known genus were assigned as "others". The proportions of these genera varied between 11.10 and 80.64% among the different samples. The top 6 most abundant genus of each group was shown in *Table 4*. These dominant genera in chicks and adults accounted together for an average of 80.04% and 12.04%, respectively *(Table 4)*. Most of the dominant genera (4/6) found in chicks were different from those of adults except the genera *Streptococcus* and *Fusobacterium*.

We employed Chao1 index and observed species curve to estimate the alpha diversity of the chicks and adults cloacal samples. The Chao1 index and observed species curve were lower in chicks than in adults samples *(Fig. 2)*, and there were significant differences ($P<0.05$) between the groups, according to Welch's t-test statistics. These results suggested that the diversity of the cloacal microbiota of adult bar-headed geese was higher than in chicks.

Bray-Curtis clustering and Venn diagrams were used to explore similarities and differences between adults and chicks *(Fig. 3)*. Analyses based on Bray-Curtis distances revealed strong clustering of the samples by ages *(Fig. 3A)*. At the OTU level, there were 186 OTUs shared between the samples from adults and chicks, whereas the other 640 OTUs and 90 OTUs, were specific to the adults and chicks, respectively *(Fig. 3B)*. These results indicated that

Table 4. *The top 6 genera in each group*

Phylum	Genus	C Group	A Group	Genus	Phylum
-	Others	20.11%	81.85%	Others	-
Fusobacteria	Fusobacterium	46.64%	6.31%	Streptococcus	Firmicutes
Proteobacteria	Psychrobacter	13.39%	2.34%	[Ruminococcus]	Firmicutes
Firmicutes	Bulleidia	8.33%	1.19%	Leuconostoc	Firmicutes
Firmicutes	SMB53	7.87%	0.88%	Oscillospira	Firmicutes
Tenericutes	Mycoplasma	1.93%	0.77%	Ochrobactrum	Proteobacteria
Firmicutes	Streptococcus	1.88%	0.55%	Fusobacterium	Fusobacteria

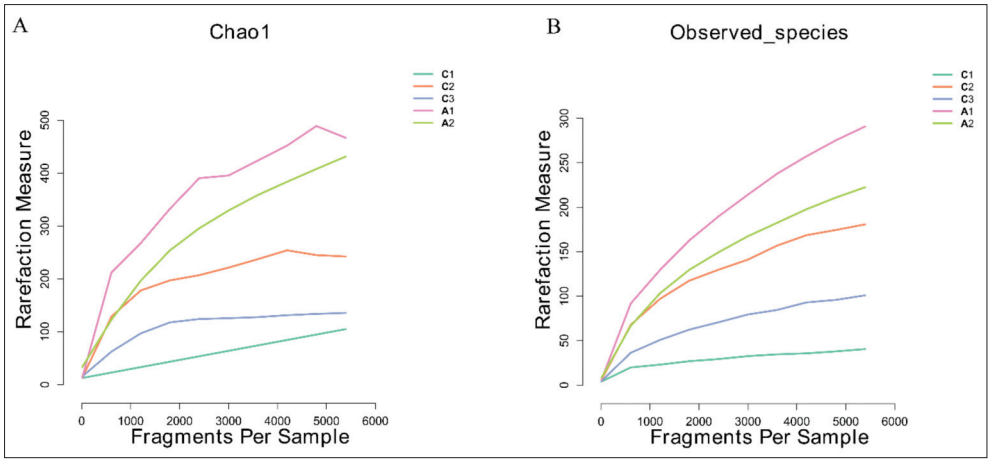

Fig 2. Comparison of the Chao1 based rarefaction curves (A) and Observed species curves (B)

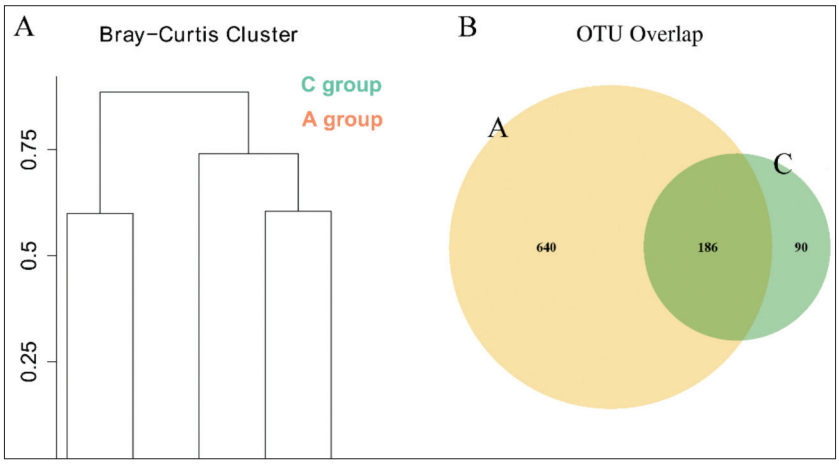

Fig 3. Hierarchical clustering of each sample (A) and the Venn diagrams (B) of both two groups

majority of OTUs (67.39%) presented in the chicks were also presented in the adults. The top 25 most abundant OTUs at the genus level shared by both adults and chicks were shown in *Fig. 4*.

DISCUSSION

In this study, we for the first time characterized and compared the cloacal bacterial microbiotas of adult and infant bar-headed geese, thus providing new insights into the impact of ages on alterations of the gut microbiotas. Our results showed that chicks had a lower and less diverse cloacal microbiota than adults. These results were consistent with earlier studies on the cloacal bacterial assemblages of both adults and chicks in a wild population of black-legged kittiwakes [33]. Our results also supported findings of newly published works by Barbosa with older penguins showing a higher diversity than younger ones [34]. There might be several reasons for age-related variations in diversity. First, the physical and chemical properties of the gastrointestinal tracts in chicks differ from adults. For example, the early colonization of the gut by facultative anaerobes, which then created the anaerobic conditions required for colonization by obligate anaerobic gut microbes [3]. Similarly, it is expected that young bar-headed geese enrich their gut microbiomes as their state of gut

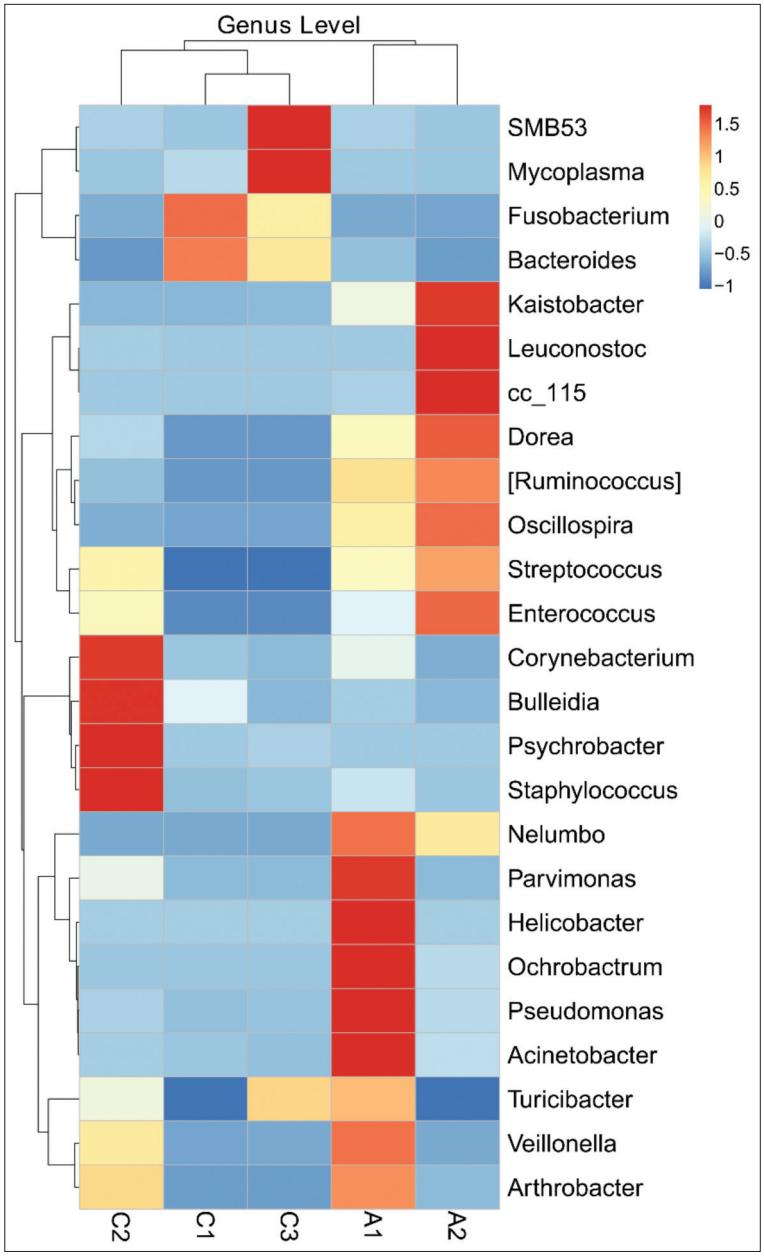

Fig 4. The heat map of the top 25 most abundant genus in each sample

transition to a stable adult state. Second, the low mobility of chicks resulted in a restricted environments from which to obtain bacteria. Therefore, their decreased microbial diversity could be related to the lower capabilities to contact with natural environments. Last, immune system was found to be one of the strongest environmental factor shaping gut microbiotas in animals [10]. Adults had developed more adaptive immune system to establish symbiotic relationship with the microbiotas compared to chicks [35]. Therefore, more microbiotas presented in the cloacal of adult bar-headed geese.

The present results clearly showed some differences in cloacal microbiotas between chicks and adults. Relative abundances of *Fusobacteria* (48.29%) and *Actinobacteria* (5.02%) in cloacal of chicks were greater than those in adults. An increase in the prevalence of *Fusobacteria* had also been reported in other birds' microbiomes such as penguins [36], emus [37] and vultures [38]. *Fusobacteria* phyla were found to be the producers of butyrate, which was known to enhance the body fat accumulation and the immune function of bird hosts [39]. In this context, we can expect that the greater relative abundance of butyrate-producing *Fusobacteria* may be helpful to increase the survival rates of chick bar-headed geese by enhancing their fat accumulation. In the case of penguins, *Actinobacteria* were found to be higher abundant in the gastrointestinal microbiotas of Adélie penguins due to the capability to degrade chitin in their diets [34]. This indicated that higher abundance of *Actinobacteria* in chick bar-headed geese may be related to their food digestion capabilities required. However, the diet composition of bar-headed geese during their early developmental stage are virtually unknown. Therefore, the diet and the microbiota of chick bar-headed geese should be integrated in future prospective studies.

Proteobacteria and *Cyanobacteria* were found to be higher in adult bar-headed geese than in chicks. The *Proteobacteria* is the largest bacterial phylum in terms of the number of culturable bacteria and is abundant in the gastrointestinal tracts in the majority of birds [14]. The observed *Cyanobacteria* correspond to the chloroplasts from the plant-based diets. As an herbivorous species, the nourishment of bar-headed geese is composed of highly fibrous plant material, mainly grass, leaves, twigs and seeds [40].

Although there were many differences between chicks and adults, our analysis of the compositions at the OTUs level in each group showed very large overlap existed in the bacterial assemblages between chicks and adults. The establishment of gastrointestinal microbiotas of young birds is characterized by a high turnover of many transient species and large changes in community structure over short periods of time [41]. For example, van Dongen et al.[33] found that, chick and adult black-legged kittiwakes shared only seven OTUs, resulting in pronounced differences in microbial assemblages. In contrast to this findings, our results showed that 67.39% of the OTUs in chicks also presented in the cloacal microbiotas of adult bar-headed geese. Given that chick bar-headed geese were fed exclusively on food regurgitated by adults, the shared OTUs could be related to the feeding habits of this bird species. Another reason might be that chicks and adults shared the same nesting environments. These 186 OTUs

shared between chick and adult bar-headed geese may be beneficial or commensal for the host, and therefore retained in the gastrointestinal tracts.

We acknowledged that our study had limitations. The sample size of adult bar-headed geese was relatively small. As such, the inability to collect required duplicate samples may reduce the accuracy of partial results. To describe the sequential changes of the cloacal microbiotas, more time points should be set in the future work.

In conclusion, this study is an elementary characterization and comparison of cloacal microbiotas in both chick and adult bar-headed geese. Future studies should include broader sampling of chicks for more detailed comparative analyses, thus help in the development of strategies to guide the formation of health-promoting microbiotas that could then be used for the artificially reared chick bar-headed geese.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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