



# Altered Gut Archaeal Communities in *Anser erythropus* Populations Wintering at Shengjin and Caizi Lakes in China

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## ABSTRACT

Archaea are an important component of the gut microbiomes of animals and play a key role in animal health. However, they have been neglected in previous microbial studies, particularly those involving migratory waterbirds. Here, we investigated the gut archaeal communities of *Anser erythropus* wintering at Shengjin and Caizi Lakes in China using metagenomic analysis of 20 fecal samples (ten samples per lake). The operational taxonomic units (OTUs) in the gut archaea of geese wintering in the two lakes represented 16 phyla, 16 classes, 24 orders, 27 families, and 45 genera, with Euryarchaeota, Thaumarchaeota, and Bathyarchaeota being the dominant gut archaeal phyla. The results of alpha diversity analysis showed a significant difference in composition between the Shengjin and Caizi Lake samples, and orthogonal partial least squares-discriminant analysis (OPLS-DA) analysis indicated that geese were well-matched with their lakes of origin. Stamp analysis revealed a difference of 27 gut archaeal species between *A. erythropus* wintering at Shengjin Lake and those at Caizi Lake. Archaeal network analysis results fell into four major modules, with *Methanobrevibacter* and *Thaumarchaeota* as the hub modules. The abundances of several bacterial and fungal genera were significantly correlated with abundances of archaeal genera in pairwise populations, and a positive correlation was observed between archaeal, bacterial, and fungal diversities in the guts of *A. erythropus* wintering at both Shengjin and Caizi Lakes ( $R=0.4$ ,  $p=2.2 \times 10^{-16}$ ;  $R=0.86$ ,  $p=2.2 \times 10^{-16}$ , respectively). This suggests that fecal bacteria and fungi may cooperate with archaea to perform crucial roles in the gut. However, because of the small sample size in this study, further studies are needed to fully investigate the altered archaea in the guts of these geese.

## Article Information

Received 08 December 2022

Revised 10 January 2023

Accepted 30 January, 2023

Available online 18 May 2023

(early access)

Published 11 June 2024

## Authors' Contribution

FSZ and GL designed the study and did the experimental work. FSZ wrote the manuscript. NX, FSZ and GL did the bioinformatic and data analysis. FSZ and NX collected the samples.

## Key words

*Anser erythropus*, Gut, Archaeal community, Diversity, Shengjin lake, Caizi lake

## INTRODUCTION

Archaea comprise a unique domain of organisms with distinct biochemical and genetic differences from bacteria and eukaryotes, and are among the least studied and least understood members of the microbiota (Baker *et al.*, 2020; Coker *et al.*, 2020). Archaea use highly diverse energy sources and have unique metabolic characteristics and cell physiology, enabling them to survive in extreme environments. These include habitats with extreme temperatures, high salinity, and extreme alkalinity and acidity (Cavicchioli, 2006; Shen *et al.*, 2008;

Andrei *et al.*, 2012; Eme *et al.*, 2018; Herbold *et al.*, 2017; Coker *et al.*, 2020). Archaea have also been detected in various non-extreme environments, including soil, oceans, lake water, and habitats associated with human and non-human animal hosts, and have been confirmed to contribute significantly to the ecological cycle (Brugere *et al.*, 2014; Blais *et al.*, 2014). While archaea only account for a very small proportion of the gut microbiome in humans and non-human animals, they have been reported to act as stable commensals of the gastrointestinal tract, participating in functions such as methanogenesis, transformation of heavy metals, trimethylamine metabolism, and immune modulation (Coker *et al.*, 2020; Deng *et al.*, 2021).

The gastrointestinal microbial community of vertebrates is the largest and most important ecosystem. Most studies of the vertebrate microbiome over the last decade have focused on bacteria, and thus little is known about the diversity and role of archaea in the gut (Peng *et al.*, 2022). However, with the advancement of research technologies and tools, a large number of archaea have recently been found in human and non-human animal gut microbial communities, and they are now considered to be

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0030-9923/2024/0004-1809 \$ 9.00/0



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an essential part of the gut microbiota along with viruses, protozoa, bacteria, and fungi (Deng *et al.*, 2021). Similar to other gut microorganisms, archaea play an important role in the maintenance of host health. For example, some species have been reported to reduce concentrations of trimethylamine-N-oxide, a harmful compound associated with several diseases, via methanogenesis (Bain *et al.*, 2006; Turnbaugh *et al.*, 2006). These methanogenic archaea can remove H<sub>2</sub>, the end-product of bacterial fermentation, to increase the efficiency of fermentation and thus improve the energy harvesting capacity of the host (Bain *et al.*, 2006; Turnbaugh *et al.*, 2006). Additionally, archaea have been associated with health conditions such as the development of anaerobic abscesses, peri-implantitis, anorexia, and inflammatory bowel disease (Nakamura *et al.*, 2010; Blais Lecours *et al.*, 2014). Archaea exhibit active metabolism and optimize the metabolic pathways of fermentative bacteria in the human gut. The composition of the gut microbiota differs among people and is dependent upon many environmental factors, including diet, chemical exposure, and use of antibiotics or other medications (Xiang *et al.*, 2019, 2021). The gut microbiota can be directly involved in the development of gastrointestinal diseases; thus, it is important to determine the composition and function of gut bacteria, fungi, and archaea in a healthy gut, as well as the alterations that cause gastrointestinal diseases, including several gastrointestinal cancers (Drancourt *et al.*, 2017).

Similar to other vertebrates, birds harbor diverse microbes within their guts, which play an important role in shaping their health (Liu *et al.*, 2020). Archaea are comparatively low in abundance in the vertebrate gut and are mostly unculturable; consequently, most data on gut archaeal diversity are derived from studies using standard universal 16S rRNA gene primers (Youngblut *et al.*, 2021). Vertebrates of all major ecological niches harbor archaea in their gastrointestinal tracts; however, little is known about archaeal diversity and community composition in the avian gut (Liu *et al.*, 2020; Youngblut *et al.*, 2021). In this study, we re-analyzed metagenomic data to investigate the diversity, composition, and potential functions of archaea in the gut of the lesser white-fronted goose, *Anser erythropus*. Using samples from geese at two wintering locations, we aimed to determine whether gut archaeal compositions exhibit the same patterns across populations. Additionally, we determined the compositional and ecological alterations of archaea in the gut of *A. erythropus* and their association with gut bacteria and fungi.

## MATERIALS AND METHODS

### *Species and study areas*

*Anser erythropus* is a species of goose belonging to

the family Anatidae and order Anseriformes (Tian *et al.*, 2021). An important indicator species in wetlands, this bird is distributed from Scandinavia to Siberia during the breeding season and winters in Japan, Korea, and China (Cao *et al.*, 2018). In recent years, these geese have experienced a continuous and rapid decline as a result of habitat loss, illegal hunting, and human disturbance. In China, *A. erythropus* prefers to winter on wetlands with typically low levels of human activity, and are mainly found in the Yangtze River floodplain, especially at Shengjin and Caizi Lakes (Yang *et al.*, 2016; Liu *et al.*, 2020). At Caizi Lake, their diet consists mainly of *Carex* species growing in mudflats, while at Shengjin Lake they mainly forage on Poaceae species on grasslands (Zhao *et al.*, 2013; Liu *et al.*, 2020).

Shengjin and Caizi Lakes (located at 30.25°–30.50°N, 116.92°–117.25°E and 30.75°–30.97°N, 117.00°–117.15°E, respectively) are typical shallow, river-connected lakes located in the middle and lower Yangtze River floodplain in Anhui Province, China. The distance between the two lakes is approximately 70 km. Both lakes are located in the humid subtropical climate zone, and function as catchment areas for Dabie Mountain and the Southern Anhui Mountains. They are also recognized as globally important stopover and wintering wetlands for migratory waterbirds on the East Asian-Australasian Flyway (Chen *et al.*, 2011; Mahtab *et al.*, 2021). Affected by rainfall and reversing river flow, the lakes show clear seasonal hydrological fluctuations. Mudflats and meadows are widely exposed during the dry season from November to April, forming suitable wintering sites for migratory waterbirds. Exposure of mudflats and meadows changes with the water levels in the lakes during winter (Yang *et al.*, 2015; Wei *et al.*, 2020). As a consequence of wetland degradation and habitat loss, the carrying capacity of these wetlands has decreased and the availability of the traditional food sources of wintering waterbirds has declined sharply (Wan *et al.*, 2016).

### *Sample collection, avian species determination, and Illumina high-throughput sequencing*

All samples were collected from Shengjin and Caizi Lakes in December 2020 using the methods described by Xiang *et al.* (2019, 2021). We selected sampling sites where only a single species was present based on synchronous waterbird surveys, and observed while geese were feeding. Using either a telescope or binoculars, we collected fecal samples left in the grass of the mudflats at the two lakes by large geese (more than 100). Fresh fecal samples were collected immediately after the geese finished foraging and defecating, with a minimum distance of 5 m between samples. To avoid cross-contamination, we changed

disposable gloves before collecting each sample, and fresh droppings were collected and stored in sterile tubes. Additionally, to minimize possible contamination from the ground, the outside of each sample was cut to avoid soil contaminants, and only the upper layer of the fecal ball was collected. Collected samples were kept in a cooler and transported to the laboratory as quickly as possible, where they were immediately stored at -80°C. The *coxI* gene of the mitochondrial genome was sequenced to confirm the avian species of origin to ensure that all fecal samples in this study were from *A. erythropus* (Xiang *et al.*, 2019, 2021). Metagenome sequencing and analysis of 20 fecal samples (10 per lake) was performed using Illumina HiSeq 2500 at OE Biotech Co., Ltd. (Shanghai, China).

#### Bioinformatics, statistical analyses, and archaea–bacteria and archaea–fungi association analyses

Metagenomic data derived from the raw sequencing analysis were converted into FASTQ format and processed using Quantitative Insights into Microbial Ecology version 2 (Xiang *et al.*, 2019). MEGAHIT (v1.1.2) software was used to perform metagenome assembly after obtaining valid reads. Clean reads were aligned against the non-redundant gene set (95% identity) using Bowtie 2 (v2.2.9). Raw reads of metagenomic data quality control were performed using the Kneaddata pipeline v0.7.2, and Kraken 2 was used to classify archaeal taxa.

We calculated alpha diversity, a measure of archaeal diversity applicable to a single sample, using the Chao1 index. Analysis of beta diversity, a measure of the degree of similarity or dissimilarity between two communities, was performed by calculating the UniFrac index using R (v3.4.1) (R Core Team, 2017). Differences in the archaeal compositions of the guts of *A. erythropus* at each lake were analyzed via partial least squares discrimination analysis (PLS-DA) using the mixOmics package in R (v3.2.1) (Rohart *et al.*, 2017). Linear discriminant analysis effect size (LEfSe) analysis was used to identify differentially abundant taxonomic features between and among groups using the non-parametric Kruskal–Wallis rank sum test. Venn analysis diagrams were created to categorize core bacteria using the “Venn diagram” package in R (v3.2.1) (Chen *et al.*, 2011). All statistical analyses and plots were generated using GraphPad Prism (v7.0). Stamp and network analyses were performed to categorize core fungal taxa using the Oebiotech tools at <https://cloud.oebiotech.cn/task/> and the Tutool platform at <https://www.cloudtutu.com/#/index>. We analyzed the relationships between archaea and fungi and archaea and bacteria using gut bacterial and fungal data from 20 fecal samples taken from Shengjin and Caizi Lakes as part of a previous study (Liu *et al.*, 2020, 2022). Pairwise Spearman correlations,

R scores, and *p*-values for operational taxonomic units (OTUs) were calculated using the Hmisc package (v.4.1.1) in R.

## RESULTS

#### Archaeal community compositions

We identified 16 phyla, 16 classes, 24 orders, 27 families, and 45 genera of archaea in the guts of *A. erythropus* wintering at Shengjin and Caizi Lakes. Euryarchaeota, Thaumarchaeota, and Bathyarchaeota were the dominant archaeal phyla, accounting for 35.41%, 27.35%, and 3.38% of the OTUs, respectively. In contrast, Heimdallarchaeota, Woesearchaeota, Crenarchaeota, Lokiarchaeota, Thorarchaeota, Micrarchaeota, and Verstraetearchaeota accounted for less than 0.02% of all archaea present in our samples (Fig. 1A). Among archaeal classes, Halobacteria, Methanomicrobia, and Methanobacteria were the most dominant, accounting for 66.41%, 15.66%, and 9.17%, respectively (Fig. 1B). Halobacteriales, Methanosarcinales, Methanobacteriales, Poseidoniales, Haloferacales, Nitrosopumilales, Thermococcales, Methanomicrobiales, Archaeoglobales, and Methanomethyliales were the dominant archaeal orders, with Halobacteriales being the most abundant, accounting for 15.03% (Fig. 1C). At the family level, Haloarculaceae, Methanobacteriaceae, Methanotrichaceae, Methanosarcinaceae, Thermococcaceae, Nitrosopumilaceae, Methanomicrobiaceae, Halobacteriaceae, Halorubraceae, and Haloferaceae were dominant, with Haloarculaceae being most abundant, accounting for 14.65% (Fig. 1D). Finally, *Halomicroarcula*, *Methanothrix*, *Methanobrevibacter*, *Nitrosopumilus*, *Micrarchaeum*, *Pyrococcus*, *Methanoculleus*, *Halorubrum*, *Salarchaeum*, and *Methanosarcina* were the dominant archaeal genera, with *Halomicroarcula* identified as the most abundant genus (14.58%) (Fig. 1E).

#### Alpha and beta diversities

Alpha and beta diversities were compared to identify and classify shifts in gut archaeal composition between the *A. erythropus* populations wintering at Shengjin Caizi Lakes. Estimates of gut archaeal alpha diversity calculated using the Simpson's and Shannon indices showed that it differed significantly between the Caizi Lake and Shengjin Lake samples, with both the Simpson's and Shannon indices being significantly higher for samples from Shengjin Lake ( $p=0.043$ ,  $0.031$ ; respectively) (Fig. 2A–B). OPLS-DA of the archaea from the guts of *A. erythropus* wintering in both lakes indicated that the samples were well matched with their lakes of origin (ANOSIM:  $p = 0.001$ ) (Fig. 3).

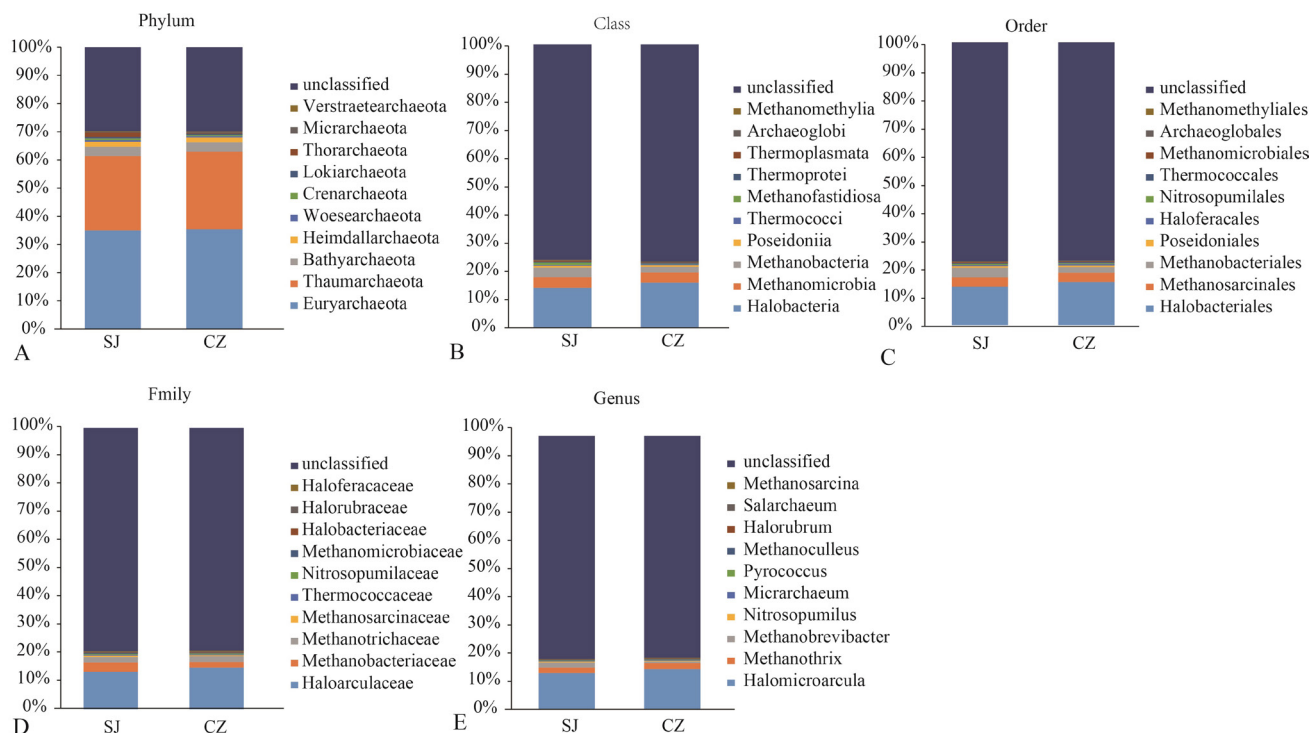


Fig. 1. Taxonomic analyses at the phylum (A), class (B), order (C), family (D), and genus (E) levels of the gut archaeal communities of *Anser erythropus* wintering at Shengjin and Caizi Lakes.

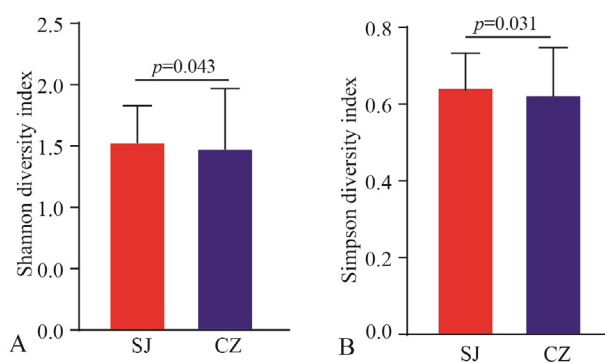


Fig. 2. Shannon (A) and Simpson's (B) indices of gut archaea diversity in *A. erythropus* overwintering at Shengjin (SJ) and Caizi (CZ) Lakes.

#### Differences in archaeal species across populations

Abundances of all gut archaeal species from *A. erythropus* wintering in Shengjin and Caizi Lakes were compared using stamp analysis. A total of 27 archaeal species differed across the guts of *A. erythropus* from the two populations (Fig. 4). All 27 of these species, including *Thaumarchaeota* archaeon, *Euryarchaeota* archaeon, *Methanosaeta harundinacea*, *Heimdallarchaeota* archaeon, *Methanosarcinales* archaeon, and *Woesearchaeota* archaeon,

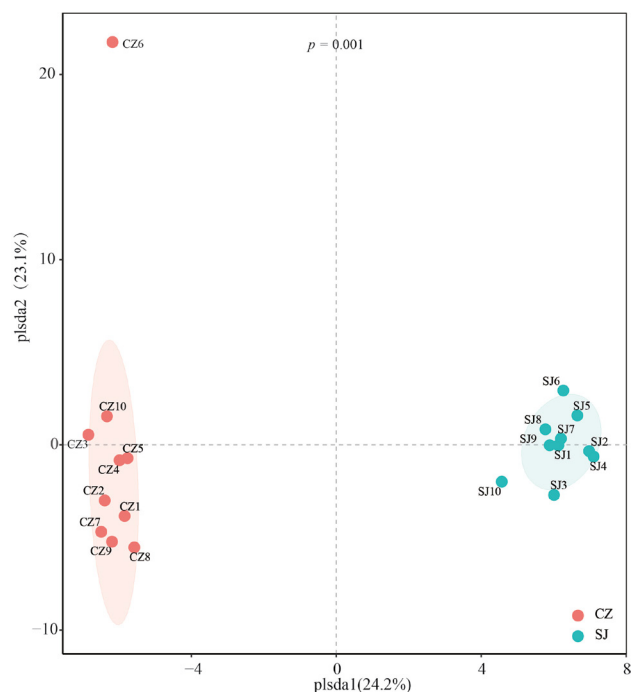


Fig. 3. OPLS-DA analysis of archaea from the guts of *Anser erythropus* wintering at Shengjin and Caizi Lakes.

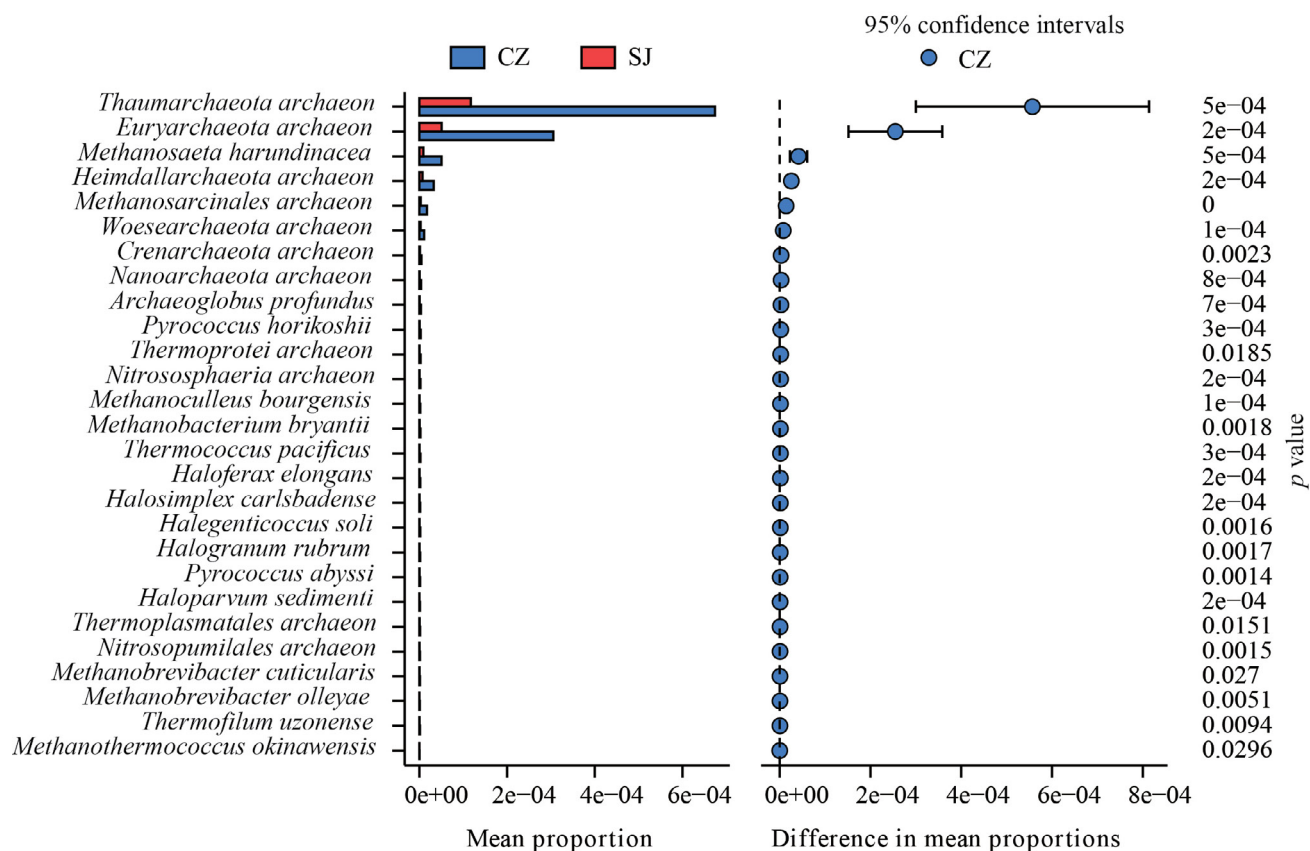


Fig. 4. Differences in abundance of archaeal species found in the guts of *Anser erythropus* wintering at Shengjin and Caizi Lakes.

had a significantly higher abundance of OTUs in samples from Caizi Lake than in those from Shengjin Lake.

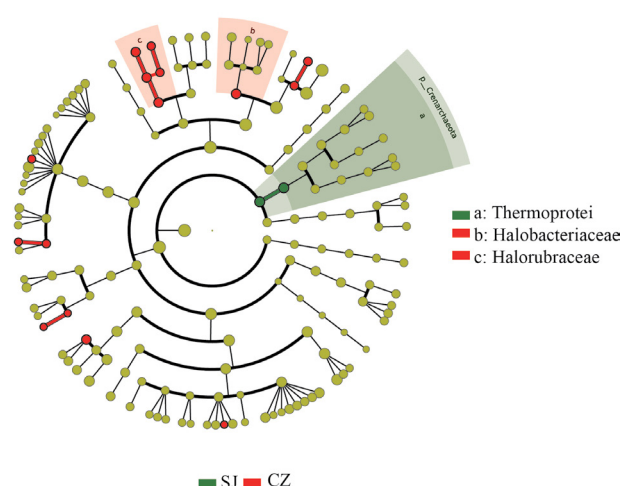


Fig. 5. Results of the linear discriminant analysis effect size (LEfSe) analysis of the gut archaea communities of *Anser erythropus* wintering at Shengjin and Caizi Lakes.

#### LEfSe and network analysis of archaea

To further explore the variation in gut archaeal community composition across *A. erythropus* populations, we performed LEfSe analysis to detect differences in the relative abundances of archaeal taxa across samples. Halobacteriaceae and Halorubraceae were significantly more abundant in *A. erythropus* wintering at Caizi Lake ( $p = 0.008$ ,  $0.001$ , respectively), whereas Thermoprotei were significantly more abundant in the geese from Shengjin Lake ( $p = 0.028$ ) (Fig. 5). Furthermore, results from our archaeal network analysis fell into four major modules, with the largest two consisting of 19 and 43 total nodes, respectively (Fig. 6). From these results, we concluded that *Methanobrevibacter psychrotolerans* was the hub of Module I, whereas *Thaumarchaeota archaeon* was the hub of Module II.

#### Bacteria–archaea and fungi–archaea associations

Abundances of some bacterial and fungal genera were significantly correlated with abundances of the archaeal genera found in our 20 samples. For example, abundances of the bacterial genera *Pseudomonas*, *Sphingobacterium*,

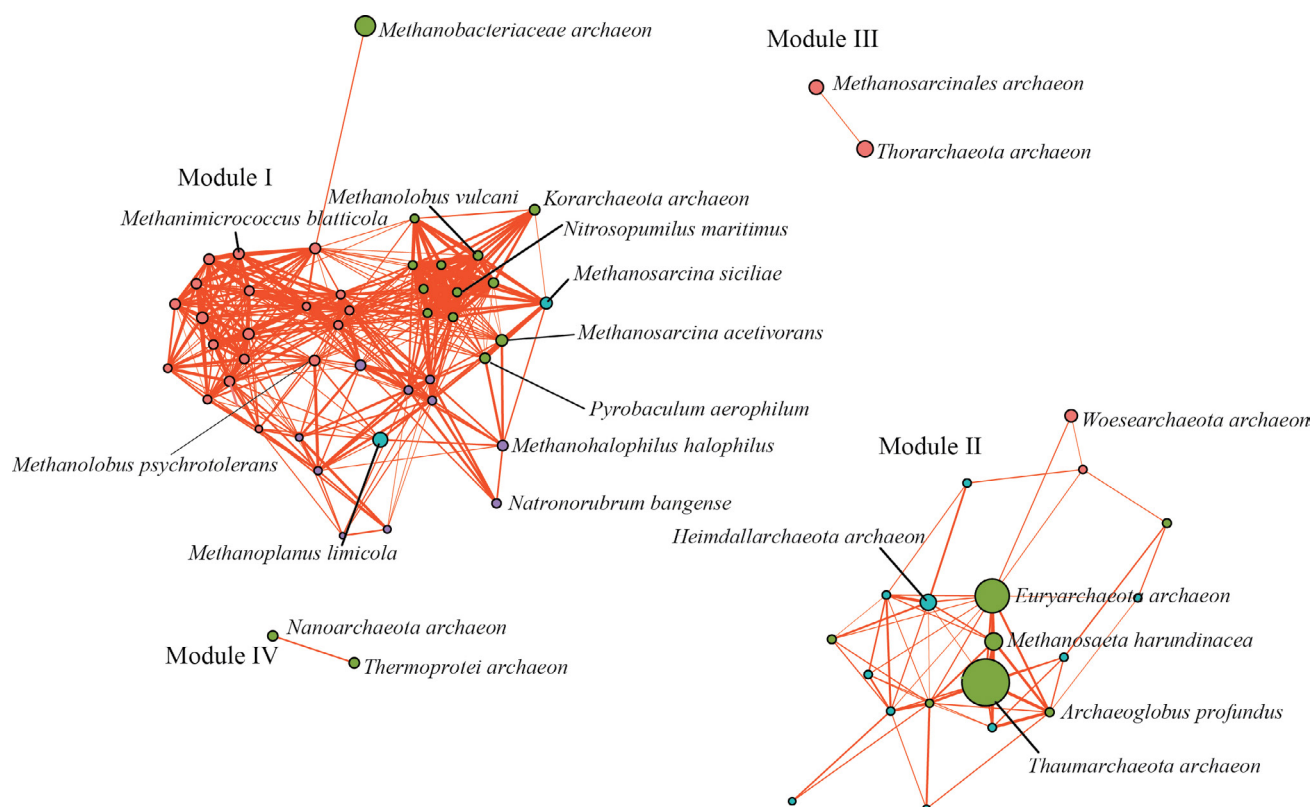


Fig. 6. Results of the correlation network analysis of the gut archaea communities of *Anser erythropus* wintering at Shengjin and Caizi Lakes.

and *Acinetobacter* were positively correlated with the abundances of most archaeal genera present ( $p < 0.05$ , 0.05, and 0.05, respectively), while abundances of *Bacillus*, *Bacteroides*, and *Parabacteroides* were negatively correlated (Fig. 7A). Additionally, a genus-level correlation heatmap for archaea and fungi revealed that the abundances of most of the top 30 archaeal genera identified were positively correlated with the abundances of most fungal genera in our samples (all  $p < 0.05$ ) (Fig. 7B).

Considering the synergistic interactions that exist among gut archaea, bacteria, and fungi in various metabolic processes, these relationships were further investigated. A positive correlation was observed between bacterial and archaeal diversities in the guts of *A. erythropus* wintering at both Shengjin and Caizi Lakes ( $R=0.4$ ,  $p=2.2 \times 10^{-16}$ ) (Fig. 7C). Archaeal and fungal diversities were also positively correlated ( $R=0.86$ ,  $p=2.2 \times 10^{-16}$ ) (Fig. 7C and 7D).

## DISCUSSION

As a domain of gut microorganisms, archaea are considered an essential group in both humans and non-

human animals. They play an important role as electron acceptors for substrates originating from anaerobic degradation of organic matter in the gut by hydrolytic and fermentative bacteria (Sereme *et al.*, 2019; Peng *et al.*, 2022). However, information on the diversity and function of archaea residing in the vertebrate gut is limited because of the unculturable nature of most taxa (Gaci *et al.*, 2014; Coker *et al.*, 2020). Compared with bacteria and fungi, very limited information is available on the metabolic pathways that define archaeal composition in birds. In this study, we analyzed next-generation sequencing data to identify archaeal diversity in the guts of *A. erythropus* wintering in Shengjin and Caizi Lakes, thus improving current understanding of the potential roles of archaea in birds.

We found that gut archaeal communities differed drastically between geese wintering at Shengjin and Caizi Lakes, with similar shifts in bacterial and fungal communities. This suggests that food resources may be an important factor influencing changes in the gut microbial community (Liu *et al.*, 2020). Previous research has shown that geese at Shengjin Lake mainly eat Poaceae, whereas geese at Caizi Lake mainly eat *Carex* species

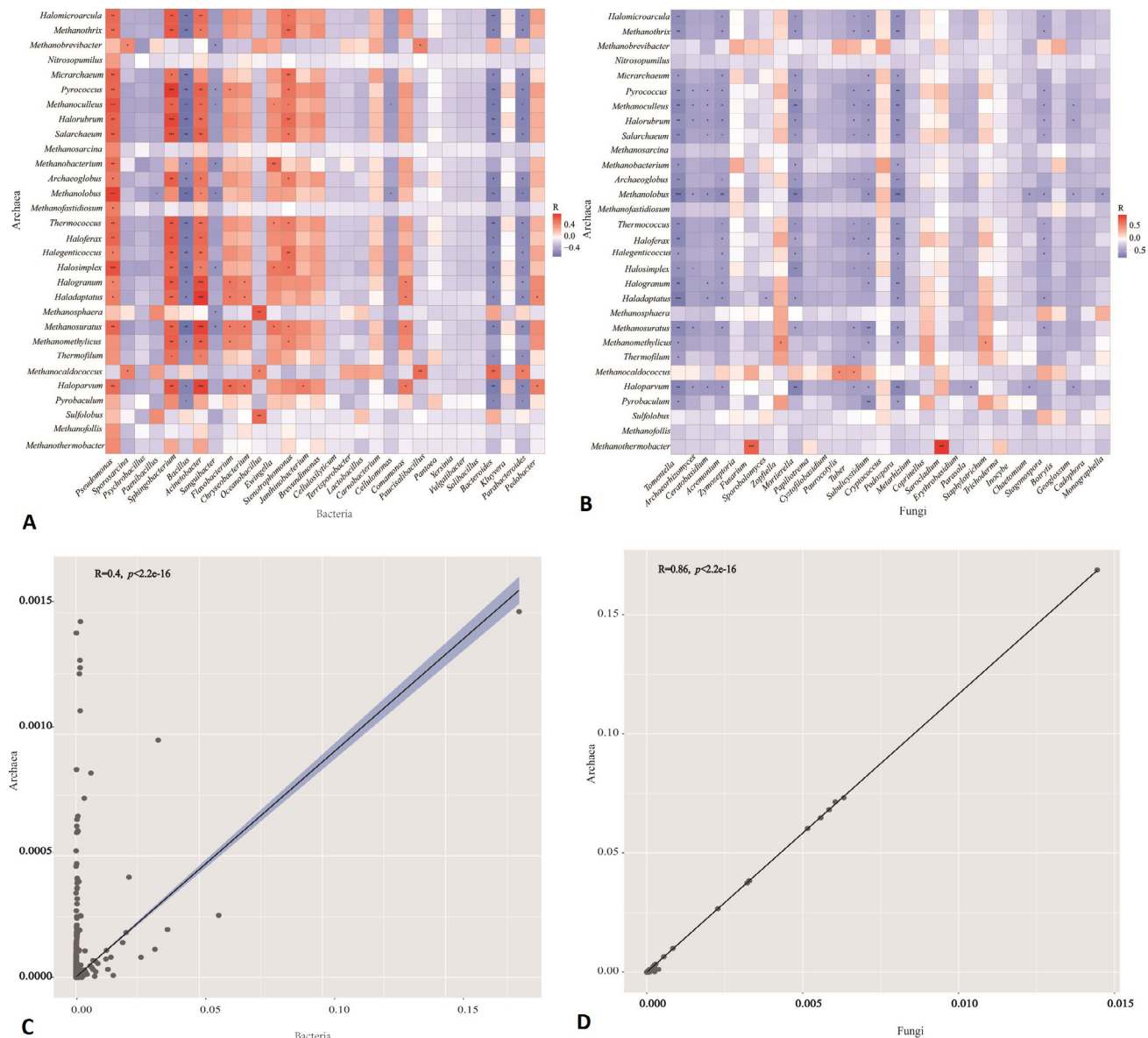


Fig. 7. Heatmaps of the top 30 archaeal-bacterial genera (A) and archaeal-fungal genera (B) associations; correlation between enriched archaea and bacteria (C), and between archaea and fungi (D) in the guts of *Anser erythropus* wintering at Shengjin and Caizi Lakes.

(Yang *et al.*, 2016). The abundance of archaea in the gut of *A. erythropus* has been shown to be significantly lower than that of both bacteria and fungi (Liu *et al.*, 2020, 2022). Analysis of the relationships between gut microbes in *A. erythropus* indicated a positive association between bacterial and archaeal diversity, with a similar relationship between archaea and fungi. This mutualism suggests that fecal bacteria and fungi may cooperate with archaea to play crucial roles in the physiology of their hosts by contributing to gut function. Some archaeal taxa,

especially methane-producing archaea, are thought to be key species that affect the composition and function of microbiota in complex host and non-host environments (Koskinen *et al.*, 2017; Moissl-Eichinger *et al.*, 2018; LeBlanc *et al.*, 2019). Additionally, some archaea are considered promising probiotic candidates, suggesting that they may play important roles in the health and disease of human hosts (Amabebe *et al.*, 2020; Coker *et al.*, 2020). We found that Euryarchaeota, Thaumarchaeota, and Bathyarchaeota were the dominant archaeal phyla in

the guts of *A. erythropus* wintering in Shengjin and Caizi Lakes. The phylum Euryarchaeota contains the greatest number and diversity of cultured lineages of archaea, many of which live under extreme conditions (Baker *et al.*, 2020). Euryarchaeota are involved in methane production and anaerobic oxidation of methane and other short-chain hydrocarbons (Orphan *et al.*, 2002; Wang *et al.*, 2019). Thaumarchaeota are among the most abundant archaea on earth and can obtain ammonia from urea and cyanate to synthesize methylphosphonate (Metcalf *et al.*, 2012; Baker *et al.*, 2020). Bathyarchaeota have also been detected in a wide range of environments, with most identified lineages living in anoxic marine, anoxic freshwater, and high-temperature hot spring locations. Evidence has shown that Bathyarchaeota can remineralize detrital proteins, possibly coupling protein degradation to hydrogen production (Lazar *et al.*, 2016). Some studies suggest that associations between archaeal taxa and animal growth could be the result of the ability of some archaea to reduce hydrogen, increasing host energy harvest and fat deposition in mouse models (Baker *et al.*, 2020; Peng *et al.*, 2022). However, this does not seem to entirely explain the effects of archaea on hosts, and the function of most archaea in birds remains largely unknown (Peng *et al.*, 2022).

In conclusion, the gut archaea of *A. erythropus* differed significantly across populations wintering at Shengjin and Caizi Lakes in China. As with variation in gut fungal and bacterial communities, differences in food resources may underly the variation seen in the gut archaeal communities of geese wintering at the two lakes. Furthermore, analysis of the relationships between microbes found in the guts of *A. erythropus* indicated a positive association between bacterial and archaeal, as well as fungal and archaeal diversity. These results suggest that fecal bacteria and fungi may cooperate with archaea to play a crucial role in the gut.

This study had some limitations. First, only 20 samples were included. Second, further investigation is required to elaborate on the functional consequences of archaea as a biomarker. Finally, more experimental technologies are needed to fully investigate the abundance and diversity correlations among bacteria, fungi, and archaea. Future studies should investigate the food resources, diet, flock size, wintering processes, and breeding/stopover site characteristics of migratory waterbirds, as well as the frequency of interactions with other fowl species (i.e., the domestic duck), as these ecological factors could have an effect on gut archaeal communities.

## ACKNOWLEDGEMENTS

The authors would like to thank Zhizhong Gong for his assistance with the laboratory work.

## Funding

This research was supported by the Natural Science Foundation for the Higher Education Institutions of Anhui Province of China (grant no. KJ2021A0246).

## IRB approval and ethical standards

No birds were harmed or killed during this study. Our methods were approved by the Animal Research Ethics Committee of Anhui Medical University (No. 81220270).

## Statement of conflict of interest

The authors have declared no conflict of interest.

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