Does gut microbiota regulate brooding in geese?

Guojun Liu1,*, Zhenhua Guo1,*,**,***, Di Liu1,*, He Meng2,**, Yuming Zheng2, Xiuhua Zhao1, Lihong Gu3, Zhifeng Chen4, Xingyong Chen3, Manyu Li1, Jinyan Sun1, Zhigang Ma4, Haijuan He1, Xiaolong Yu1 and Fanghong Hu6

1 Heilongjiang Academy of Agricultural Sciences, Animal Husbandry Research Institute, No. 368 Xuefu Road, Harbin 150086, P.R. China
2 Department of Animal Science, School of Agriculture and Biology, Shanghai Jiao Tong University; Shanghai Key Laboratory of Veterinary Biotechnology, Shanghai 200240, P.R. China
3 Hainan Academy of Agricultural Sciences, Institute of Animal Science and Veterinary Medicine, 14 Xingdan Road, Qiongshan District, Haikou, 570203, P.R. China
4 Heilongjiang Academy of Agricultural Sciences, Qiqihare Branch Academy, No. 2 Heyi Road, Qiqihare 161005, P.R. China
5 College of Animal Science and Technology, Anhui Agricultural University, No. 130 Changjiangxi Road, Hefei, 230036, P.R. China
6 Agricultural and Rural Bureau, Longhexi Road, Lian, 237006, P.R. China

Submitted: March 15, 2021. Final revision received: June 28, 2021. Accepted: August 30, 2021

Abstract
Domestic geese can reduce the amount of food intake when brooding. Because of the reduction in food intake, the total number of microorganisms in the gut is also reduced. Will this affect the goose’s thinking and make the goose stop brooding and eat food? We hypothesize that gut microbiota affects the brain through a brain–gut peptide and further regulates the breeding behavior of geese. In this study, we evaluated the microbiome related to the goose and transcription groups of brooding and egg production periods. The changes and differences in gut microbiota and gene expression of female geese in different reproduction periods were analyzed, and the possible interaction between them was explored. The results showed that the relative abundance of Faecalibacterium with a growth-promoting effect in the cecum was higher in the egg production group than in the brooding group. Microbial metabolic pathways with significant differences between the two groups were also enriched in the secondary functional groups with different gut microbiota metabolism. The downregulated genes in the egg production group were mainly related to energy metabolism, such as ATP synthesis-related genes. These results suggest that the brooding group’s gut microbiota can make relevant changes according to the

* Zhenhua Guo and Guojun Liu contributed equally to this work and are considered equal first authors.
** Corresponding authors; e-mails: gzhh00@163.com; liudi1963@163.com; menghe@sjtu.edu.cn
*** ORCID: 0000-0003-0592-5988

© Koninklijke Brill NV, Leiden, 2021 DOI 10.1163/15707563-bja10059
reproduction stage of the goose. Since the amount of food taken in is reduced, it can promote the decomposition of the host’s fat. Simultaneously, insulin is also used to deliver messages to the brain; it is necessary to end the brooding behavior at an appropriate time and for eating to start.

Keywords
Brooding; goose; gut microbiota; microbiome; transcription group

Introduction

The theory that gut microbiota can manipulate our social actions for its own benefit is getting research attention (Bauer et al., 2016; Amato et al., 2017; Muller et al., 2020; Wang et al., 2020a). Domestic geese can reduce the amount of food intake when brooding. Because of the reduction in food, the total number of microorganisms in the gut is also reduced. Will this affect the goose’s thinking and make it stop brooding and start eating food?

The mutualism established between the gut microbiota and the host has reached a relatively steady state in the long process of coevolution. The composition and distribution of gut microbiota seem to be ruled by reproducible laws in each life stage of the host (Meng et al., 2014; Hou et al., 2016; Ding et al., 2017). Goose gut microbiota actively participates in the host’s whole life process and interacts with it (Li et al., 2020; Wang et al., 2020b).

Brooding is common in birds’ reproduction behavior, and birds in the brooding stage will stop laying eggs and put energy into nesting and hatching (Yu et al., 2016). Of all domestic poultry, the goose has the strongest brooding ability (Liu et al., 2018a). The average annual egg production of a goose is only 30–40 eggs, and the annual egg production of a goose is generally no more than 100 eggs. However, the annual egg production of today’s egg-producing chickens has reached about 300 eggs. Therefore, it is a prerequisite for large-scale production to improve the breeding behavior of the goose and improve its reproductive performance (Zhang et al., 2019).

The reproductive activity of a goose is regulated by the hypothalamus–pituitary–gonad axis (HPGA), resulting from multiple factors such as genes and the environment (Liu et al., 2018a). A previous study has shown that gut microbiota plays a role in the setting of the intestinal circadian rhythm (Kuang et al., 2019). Our early study has shown that the goose’s egg-laying behavior is closely related to the circadian rhythm (Liu et al., 2020). The HPGA feedback changes in response to the external environment and regulates the goose’s reproductive behavior through the endocrine system (Yuan et al., 2020). At present, the academic community has reached a consensus on the effect of sex hormones on the brooding behavior in geese. However, there is still a lack of research on gene expression related to goose brooding behavior outside of sex hormones.

This study measured the microbiome and transcription group related to Western Anhui white geese in the brooding and egg production periods. The changes
Figure 1. Relationship between gut microbiota and goose brooding behavior. During brooding, the food intake decreased, and the source of gut microbiota-degradable substrates decreased. Gut microbiota transmit signals to the hypothalamus through brain–gut peptides and to communicate the need to end the brooding behavior. The hypothalamus is regulated by the hypothalamus–pituitary–gonad axis (HPGA) to end goose brooding behavior.

and differences in gut microbiota and gene expression of female geese in different reproduction periods were analyzed, and the possible interaction between them was explored. This study aimed to clarify the role of gut microbiota in a goose’s reproductive activities (fig. 1).

Materials and methods

Animals

The experimental geese were Western Anhui white geese, which came from Lu’an, Anhui Province. There were 2500 geese in total, and the specific methods of feeding and management were described in our previous report (Liu et al., 2018b). The feed formulated for the egg-laying period included corn 63%, soybean meal 25.3%, fish meal 1.0%, methionine 0.06%, limestone 7.7%, calcium hydrogen phosphate 1.7%, and salt 0.26%, with an energy content of 11.3 MJ/kg. The Western Anhui white geese were divided into brooding (B) and egg production (C) groups. At the age of 300 days, five brooding groups and five egg production groups of female
geese were randomly selected. The geese were slaughtered, and the corresponding samples were obtained. The study was approved by the Committee for Animal Welfare of the Institute of Animal Husbandry of HAAS, Heilongjiang, China, No. NKY-20140506, Ministry of Science and Technology.

Gut microbiota sample preparation and sequencing

The intestinal contents [jejunum (K), cecum (M), and manure (F)] were collected after slaughter and stored at −20°C, and sent to Personalbio Co., Ltd. (Shanghai P.R. China) for sequencing. Primers (F: 5′-AYTGGGYDTAAGNG-3′, R: 5′-TACNGGGTATCTATCC-3′) were used to amplify the V4 hypervariable region of the 16S rRNA gene (Zhao et al., 2013).

Gut microbiota data analysis

QIIME (Quantitative Insights into Microbial Ecology, v1.8.0, Seattle, WA, USA) was used to merge operational taxonomic units (OTUs) according to partition sequence similarity. Greengenes (release 13.8, San Francisco, CA, USA; https://greengenes.secondgenome.com) was the template sequence for OTU taxonomic status identification. The function of the metabolic pathway was predicted by STAMP (Halifax, NS, Canada) software (Parks et al., 2014).

Sample collection and sequencing of transcription group and data analysis

The five brooding groups and five egg production groups of female geese are described above. The pituitary samples, thalamus, and ovary were collected (Liu, 2018b) and stored in cryopreservation tubes at −80°C for RNA extraction and sequencing (Illumina HiSeq 2500, Illumina, Inc. San Diego, CA, USA). The reference genome information used in this project was NCBI: GCF_000971095. The samples were analyzed by expression difference analysis, enrichment analysis, and cluster analysis.

Results

Gut microbiota data analysis

Diversity index. The first five bacterial sequences of gut microbiota at the phylum level were the same, but there were significant differences (fig. 2A). There was a great difference at the genus level (fig. 2B), and there was a significant difference between the Chao1 and ACE indices of the jejunum microbiota (table 1).

The genus-level linear discriminant analysis effect size (LEfSe) analysis results showed a significant difference in the classification units between brooding and egg production groups in jejunum and manure. The annotated classification units in jejunum were Ruminococcaceae, Staphylococcaceae, Leuconostocaceae, Deltaproteobacteria, Desulfovibrionales, Desulfovibrionaceae, Desulfovibrionaceae, Desulfovibrionaceae,
Figure 2. Community composition and abundance distribution. Phylum level (A) and genus level (B).

Paraprevotellaceae, and *Jeotgalicoccus*. All of the above classification units were observed in the egg production group, and their relative abundance significantly was improved (fig. 3A). The results showed that the relative abundances of *Leuconostocaceae* in the egg production group were higher than in the brooding group. The relative abundances of *Mycoplasma*, *Caloramator*, and *Clostridium* in the egg production group were significantly higher than in the brooding group (fig. 3B).

Studies have shown there is a difference between the brooding group and the egg production group in relative abundance at the genus level. We found that the relative abundance of *Faecalibacterium* in the cecum was significantly lower in the brooding group than in the egg production group (fig. 3C), while the relative
Table 1. Microbiome diversity index.

<table>
<thead>
<tr>
<th>Site</th>
<th>Index</th>
<th>BK</th>
<th>CK</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jejunum</td>
<td>Simpson</td>
<td>0.81 ± 0.11</td>
<td>0.81 ± 0.2</td>
<td>0.958435</td>
</tr>
<tr>
<td></td>
<td>Chao1</td>
<td>557.86 ± 62.85</td>
<td>720.08 ± 108.77</td>
<td>0.025827</td>
</tr>
<tr>
<td></td>
<td>ACE</td>
<td>579.47 ± 65.7</td>
<td>756.09 ± 106.86</td>
<td>0.01733</td>
</tr>
<tr>
<td></td>
<td>Shannon</td>
<td>4.76 ± 0.88</td>
<td>5.08 ± 1.39</td>
<td>0.672612</td>
</tr>
<tr>
<td>Cecum</td>
<td>Simpson</td>
<td>0.95 ± 0.05</td>
<td>0.98 ± 0.02</td>
<td>0.268578</td>
</tr>
<tr>
<td></td>
<td>Chao1</td>
<td>1293.38 ± 616.51</td>
<td>1515.25 ± 330.79</td>
<td>0.504341</td>
</tr>
<tr>
<td></td>
<td>ACE</td>
<td>1327.63 ± 628.8</td>
<td>1552.42 ± 344.75</td>
<td>0.508769</td>
</tr>
<tr>
<td></td>
<td>Shannon</td>
<td>7.15 ± 1.79</td>
<td>8.05 ± 0.7</td>
<td>0.340805</td>
</tr>
<tr>
<td>Manure</td>
<td>Simpson</td>
<td>0.96 ± 0.03</td>
<td>0.93 ± 0.05</td>
<td>0.31059</td>
</tr>
<tr>
<td></td>
<td>Chao1</td>
<td>1026.83 ± 239.12</td>
<td>927.3 ± 256.58</td>
<td>0.543514</td>
</tr>
<tr>
<td></td>
<td>ACE</td>
<td>1064.45 ± 233.87</td>
<td>958.48 ± 276.05</td>
<td>0.531352</td>
</tr>
<tr>
<td></td>
<td>Shannon</td>
<td>7.03 ± 1.03</td>
<td>6.53 ± 0.93</td>
<td>0.439157</td>
</tr>
</tbody>
</table>

abundance of *Clostridium* in the brooding group was significantly higher than in the egg production group (fig. 3D). In the brooding group’s manure, the relative abundance of *Bacteroides* was significantly higher than in the egg production group
The relative abundance of Enterobacteriaceae in the brooding group’s manure was significantly lower than in the egg production group (fig. 3F).

**Principal components analysis.** The results of the principal components analysis (PCA) are shown in supplementary fig. S1A–C. The jejunum and cecum were divided into two groups. However, their confidence intervals still overlapped and cannot be completely separated into two groups. The differences between the gut microbiotas were the smallest in the manure.

**Microflora metabolic function prediction.** The secondary functional groups of microorganisms were clustered and analyzed. There were significant functional groups in the jejunum and cecum and no significant functional groups in manure, consistent with the PCA results. As shown in supplementary fig. S2A, B, regarding the biodegradation and metabolism of the xenobiotics in the jejunum, the brooding group’s lipid metabolism was higher than that in the egg production group. Furthermore, the brooding group showed a lower biosynthesis of other secondary metabolites than the egg production group. Moreover, the brooding group displayed higher cecum folding, sorting, and degradation than the egg production group. Finally, the brooding group showed a lower energy metabolism than the egg production group.

**Transcription group analysis**

**Differential expression analysis.** Supplementary fig. S3 shows the differential gene expression of the brooding and egg production groups. The differentially expressed genes in the pituitary were the most abundant. The upregulated genes included G protein–coupled receptor synthesis (GPR12, GPR157, GPR171, and GPR65), Rho GTPase activator protein synthesis (ARHGAP15, ARHGAP20, ARHGAP24, and ARHGAP6), sialic acid transferase synthesis (ST6GALNAC1, ST6GALNAC2, ST8SIA4, and ST8SIA5), calcium transport regulation (CARHSP1, CRACR2B, CAMK1G, and CAMK4), gonadotropin-releasing hormone receptor synthesis (oCA 106048650), progesterone receptor synthesis (PGR, PAQR9), thyrotropin-releasing hormone receptor synthesis (TRHR), and many genes related to material transport and cell cycle regulation.

The downregulated genes included ATP synthesis (ATP50, ATP5G1, ATP5H, and ATP5I), NADH oxidoreductase synthesis (NDUFAF4, NDUFA1, NDUFA12, and NDUFA2), cytochrome synthesis and oxidase synthesis (LOC106032950, LOC106035251, LOC106047738, and LOC106042267), mitochondrial ribosomal protein synthesis (MRPL16, MRPL17, MRPL23, and MRPL33), follistatin-like protein synthesis (FSTL4), melanin-concentrating hormone receptor synthesis (MCHR2), calcitonin receptor synthesis (CALCR), prolactin (PRL), and other related genes.

The function of differentially expressed genes in the thalamus was similar to that in the pituitary. Upregulated genes included G protein-coupled receptor related genes (GPR18, GPRC5 B, and RGS8), calcium synthesis-related genes (C2, CD2, CDH19, and CASQ2). Downregulated genes included those involved in NADH...
oxidoreductase synthesis, cytochrome oxidase synthesis, mitochondrial ribosomal protein synthesis, and other related genes.

There were fewer ovarian differentially expressed genes. Upregulated genes included solute carrier family genes (LOC106031470, LOC106049801, SLC25A32, and SLC34A1), luteinizing hormone/chorionic gonadotropin receptor (LHCG), and transport genes. Downregulated genes included those for cytochrome oxidase synthesis, ribosomal protein synthesis, and other related genes.

Enrichment analysis of differentially expressed genes via KEGG. Figure S4A–C shows that the genes expressed in the pituitary, thalamus, and ovary significantly enriched different KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways. The pituitary had six significantly different KEGG pathways, and the thalamus had five significantly different KEGG pathways. The ovary did not show significantly different KEGG pathways.

Discussion

The diet causes differences in the gut microbiota (Sonnenburg et al., 2016). Geese reduce food intake during periods of brooding, and this study showed that, whether at the phylum level or at the genus level, the gut microbiota of the brooding and egg production groups were different. Much of the gut microbiota comprised common symbiotic bacteria and probiotics. Interestingly, at the phylum level, the top five bacterial relative abundances were consistent, but this was not so at the genus level, which confirmed that the diet causes differences in the gut microbiota. In 2018, when our experiment started, we were still using the V4 hypervariable region for analysis. Now we know that it is not accurate to annotate taxonomy to the genus level only by the V4 hypervariable region of the 16S rRNA gene. This method has some limitations and may lead to inaccurate results.

The LEfSe analysis results showed that Desulfovibrio, Leuconostoc, and Jeotgalicoccus were relatively abundant in the jejunum of the egg production group. Desulfovibrio, which provides electrons for organic substrates, is one of the significant sulfate-reducing bacteria in the digestive tract and is associated with intestinal inflammation (Chen et al., 2019). It is a biomarker of gestational diabetes (Crusell et al., 2018). Leuconostoc is one of the intestinal probiotics and plays an active role in the recovery of diarrhea (Fijan et al., 2014). Jeotgalicoccus is often observed in aquatic organisms. The brooding group’s manure had a higher relative abundance of Mycoplasma, Caloramator, and Clostridium. Mycoplasma is abundant in aquatic organisms and symbiotic with the host (Lian et al., 2020). It provides riboflavin, sugar, and amino acids to the host (Wang et al., 2016), which affect host metabolism. Caloramator survives at high temperatures, promoting metabolism and modifying carbohydrates (Patel et al., 2016). When brooding occurs, the body temperature of geese is higher than when they are not brooding. Caloramator may also be a biological marker of the gut microbiota of the reproduction period of the goose population. Clostridium can help Treg cells in the intestinal mucosa to resist
inflammation (Nagano et al., 2012). *Clostridium* occurs more frequently during pregnancy after antibiotic treatment (Ianiro et al., 2016). *Clostridium* also regulates insulin resistance and reduces glucose uptake (Salles et al., 2020).

Our results revealed a significant difference between *Faecalibacterium* and *Clostridium* in the cecum. The relative abundance of *Faecalibacterium* is high in the egg production group, promoting growth (Liu et al., 2021). It is an important probiotic, one of the main butyrate producers in the gut, and helps the host resist inflammation (Ganesan et al., 2018). The relative abundance of *Clostridium* is higher in the brooding group.

From the secondary functional groups with differences in gut metabolism, the metabolic pathways with significant differences between the two groups were also enriched in energy metabolism and substance synthesis. The jejunum was mainly responsible for nutrient absorption, xenobiotics biodegradation, and metabolism. Levels of lipid metabolism secondary functional groups in the brooding group were higher than in the egg production group, indicating that the food stored in the open field is being used as efficiently as possible. In the biosynthesis of other secondary metabolites, the brooding group shows a lower synthesis rate than the egg production group and tries converting food into energy to prevent fat deposition, indicating that the jejunum gut microbiota has passed signals to the host during brooding and requires food intake. The cecum is mainly responsible for storing energy, and metabolism in the brooding group was lower than in the egg production group. Differences in lipid metabolism gene expression were also detected in our results, indicating that the cecum microbiota of the brooding group attempts to reduce the level of the host’s energy metabolism. Our PCA results showed that the flora of the jejunum, cecum, and manure could not be completely divided into two groups. This suggests that the gut microbiota is more or less the same between the breeding group and the egg production group.

The endocrine system of the brooding group contrasted significantly with that of the egg production group, indicating that the brooding group’s endocrine level has changed. The difference in gene expression in this study proved this. Concerning the site of expression in the tissue, in the pituitary the difference between female geese of the brooding group and of the egg production group was the largest, followed by the thalamus and the ovary, which had similar differences. The difference in gene expression in the ovary was the smallest, which was confirmed by the KEGG enrichment analysis. Therefore, we assume that gut microbiota affects the brain through brain–gut peptides, and further that the HPGA regulates goose brooding behavior.

The brooding behavior of poultry is mainly related to various hormones and neurotransmitters produced by the hypothalamus and pituitary, and this mechanism has been widely demonstrated in many poultry species (Zhu et al., 2019). However, in addition to controlling hormone secretion and other endocrine-related genes, this study also noted significant differences in energy metabolism, substance synthesis, transport, and the cell cycle between brooding and egg production stages. The
genes upregulated in the egg production group and the brooding group were mainly related to material transport and cell cycle regulation, such as genes related to G protein-coupled receptor synthesis (\textit{GPR12} and \textit{GPR18}). These genes are associated with the regulation of light perception, the immune system, and the autonomic nervous system in the body, which can maintain homeostasis, and promote cell growth, division, secretion, and metabolism (Lu et al., 2012).

Rho GTPase activator protein synthesis-related genes (\textit{ARHGAP15} and \textit{ARHGAP20}) promote cell division and tumor formation (Pedersen & Brakebusch, 2012; Ridley, 2012). There are also genes related to calcium transport regulation and calcium-containing substance synthesis (\textit{CAMH4} and \textit{CASQ2}) that are closely related to egg formation (Royer & Rios, 2009). Similarly, in addition to hormone secretion regulation-related genes, the genes downregulated in the egg production group compared with the brooding group are mainly associated with energy metabolism, such as ATP synthesis-related genes (\textit{ATP5I} and \textit{ATP5G1}), which express ATP synthase complexes (Maak et al., 2001). Genes related to oxidoreductase synthesis (\textit{NDUFA1} and \textit{LOC106032950}) express and direct a series of enzymes related to cell respiration (Urbanová et al., 2017; Katsyuba et al., 2018). Mitochondrial ribosomal protein synthesis-related genes (\textit{MRPL16} and \textit{MRPL17}) control mitochondrial ribosomal subunit production and further affect cell energy metabolism (Smagin et al., 2018; Ózsvári et al., 2020).

The difference in the signal pathway of KEGG enrichment analysis was consistent with the difference in gene expression. Taken together, the more differentially expressed genes in the egg production group reflect the laying behavior. The more differentially expressed genes in the brooding group focused on the control of energy metabolism and physiological processes, promoting energy production in the body and egg brooding, as well as supplementing the energy that geese lack as a result of reduced eating when nesting. The above results suggest that the brooding group’s gut microbiota can make relevant changes according to the stage of goose digestion. Since food intake decreases, it stimulates the host to decompose fat. Simultaneously, insulin delivers information to the brain prompting the goose to end the brooding behavior and eat food.

**Conclusion**

It can be assumed that gut microbiota, which is maintained during brooding, does not have a source of decomposable substrate and decomposes gut cells to provide energy; otherwise, gut microbiota will remain dormant due to a lack of substrate. Moreover, the host (goose) will go extinct if it is unable to maintain a longer brooding state. Thus, to obtain a balanced state, brooding will continue for some time, during which gut microbiota will affect the host (goose) and prompt it to end brooding, promoting food intake; however, the importance of the role gut microbiota plays in promoting food intake in the host (goose) needs further research.
Acknowledgements

This work was supported by the China Agricultural Research System (CARS-42-24) and the Heilongjiang Academy of Agricultural Sciences Crossing Project (HNK2019CX18). We would like to thank LetPub (www.letpub.com) for its linguistic assistance during the preparation of this manuscript.

Competing interests

The authors declare that they have no conflicts of interest to report.

Supplementary material

Supplementary material is available online at: https://doi.org/10.6084/m9.figshare.16538124

References


