Heat stress affects duodenal microbial community of indigenous yellow-feather broilers as determined by 16S rRNA sequencing

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**ABSTRACT**

The present study was conducted to investigate the effects of heat stress on microbial community in the duodenum of yellow-feather broiler chickens based on 16S rRNA sequencing. A total of 40 female Chinese indigenous yellow-feather broilers (56-day-old, average initial body weight of 840.75 ± 20.79 g) were randomly allocated to two groups, including the normal treatment group (NT group, 21.3 ± 1.2 °C, 24 h/day) and the heat stress group (HS group, 32.5 ± 1.4 °C, 10 h/day), and the relative humidity (RH) of both two groups was maintained at 65 ± 5%. The experiment conforms to a completely randomised trial design. Broilers in both of two groups were fed basal diet, each group consisted of five replicates, and four broilers in each replicate. The feeding trail lasted 4 weeks. The results showed that although the broilers in the HS group had lower abundance-based coverage estimators (ACE) and Chao1 richness estimator (Chao1) index of duodenal microbial, there were no significant differences in duodenal microbial alpha diversity index between NT and HS groups, including ACE, Chao1, Shannon and Simpson index (p > .05). Regarding the microbial composition and abundance, at the phylum level, heat stress exposure significantly decreased the relative abundance of Bacteroidetes in duodenum compared with NT group (p < .05). At the genus level, compared with the NT group, heat stress exposure significantly reduced the relative abundance of Cupriavidus, Leptothrix, Janthinobacterium, Pseudomonas, Stenotrophomonas, Desulfovibrio, Oscillospira and Dorea in the duodenum (p < .05). In conclusion, heat stress affected the duodenal microbial community in indigenous yellow-feather broilers.

**HIGHLIGHTS**

- Heat stress had adverse effects on duodenal microbial composition of Chinese indigenous yellow-feather broilers.
- Strategies that improve the duodenal microbial balance may promote the gut health and reduce the economic losses caused by heat stress in broiler production.

**Introduction**

Heat stress, as one of the environmental stressors, caused serious impact on broilers industry and cannot be ignored in tropical and subtropical regions (Furlan 2004; Alhenaky et al. 2017; He et al. 2018a). Due to the feather and high-density farming, broilers are extremely sensitive when confronting heat stress also results in high mortality (Khosravinia 2015; Habashy, Milfort, Adomako et al. 2017; Iyasere et al. 2021). The detrimental effect of heat stress on growth performance, endocrine regulation and immune function was found in previous studies (Tu et al. 2016; Habashy, Milfort, Fuller et al. 2017; Punchihewage et al. 2018; Abo Ghanima et al. 2020; Liu, Ou et al. 2021). Furthermore, inflammation and disruption in intestinal barrier caused by heat stress were also reported in broilers (Quinteiro-Filho et al. 2010; Kikusato et al. 2021), which lead to the injury of intestinal morphology and mucosa integrity (Liu, Zhu et al. 2021; Porto et al. 2015). In recent years, intestinal health has received increasing attention and was presumed as an important indicator of broiler’s health (Liu, Guo et al. 2020). The intestine has digestive, absorptive, metabolic,
The gut microbiome is easily affected by nutrition and environment. Huaixiang chicken is a popular chicken breed among the Chinese indigenous yellow-feather broilers, and they are widely farmed in Southern China (Liu et al. 2019; Guo et al. 2021), so the high temperature in tropical can deeply affect the Huaixiang chicken’s industry. Duodenum is the beginning of small intestine and can receive the bile that was produced from liver to perform digestion and detoxification function (Gass 2007; Khan 2011). In recent years, research about the effects of heat stress on gut microbes has mainly focused on the mid-gut, such as ileum and caecum. The highly concentrated bile makes the duodenum different from ileum and caecum when it is affected by heat stress. However, there are extremely limited studies regarding the impacts of heat stress on duodenal microbiome in indigenous broilers. Hence, this research was performed to evaluate the role of heat stress in indigenous broiler’s duodenal microbial community based on 16S rRNA sequencing.

### Materials and methods

#### Chickens and management

A total of 40 56-day-old Huaixiang chickens (Chinese indigenous yellow feather broilers) were randomly allocated to 2 treatment groups (NT group: Normal treatment; HS group: Heat stress) with 5 replicates of 4 birds each. To exclude the influence of gender, 40 female broilers were selected in this experiment. The initial body weight of birds was 840.75 ± 20.79 g and was kept at 65 ± 5%. The average temperature of the NT group was maintained 21.3 ± 1.2°C 24 h/day and the HS group was about 32.5 ± 1.4°C 10 h/day (8:00 am–18:00 pm), and the rest time of HS group was consistent with the ambient temperature of the NT group. For each group, birds were free accessed to feed and water and were kept in stacked cages. The basal diet including nutrient level and ingredient composition are described in Table 1 and it was formulated according to the Feeding Standard of Chinese Chickens (NY/T33-2004). The feeds were given to the broilers in mash form. The light: dark cycle maintained for 10:14-h and relative humidity (RH) was kept at 65 ± 5%. The average initial body weight of birds was 840.75 ± 20.79 g and there were no significant differences of initial body weight between NT and HS groups. The Animal Ethics Committee of Guangdong Ocean University (Zhanjiang, China) has approved the protocol of animal feeding and care involved in this experiment.

#### Total DNA extraction

All broilers were fasted 24 h for sampling at the end of trial. One broiler was randomly selected from each replicate (n = 5 per group), and then jugular vein bleeding and slaughtered. We selected the U-shaped curved intestine behind the masticatory stomach as the duodenum. The duodenum was separated, and washed by ice-cold saline, and then small intestinal mucosa were scraped with sterile glass slide, and frozen in the liquid nitrogen immediately before DNA extraction.
The OMEGA DNA isolation kit (D5625-01; Omega, Norcross, GA) was used to extract the total microbial genomic DNA samples from the duodenum. The quality of the DNA was assessed by 0.8% agarose gelelectrophoresis and ultraviolet spectrophotometry. All extracted DNA samples were stored at −20°C before further analysis.

Preparation of library and Illumina MiSeq sequencing

Preparation of library and Illumina MiSeq sequencing was managed by Personalbio. (Shanghai, China). To prepare the library, the specific sequences that can reflect the composition of the microflora were amplified by polymerase chain reaction (PCR) with the sample-specific Barcode sequence. (Barcode sequence was added to generate indexed libraries that were ready for downstream NGS on the MiSeq platform.) And the Q5 high-fidelity DNA polymerase which provided by the NEB company was used for PCR amplification. The amplified products were checked by the 2% agarose gel electrophoresis and the target fragments were retrieved by the Gel Recovery Kit for AXYGEN Company (Union City, CA). According to the result of agarose gel electrophoresis, we used the fluorescent reagent (Quant-iT PicoGreen dsDNA Assay Kit) and the quantitative instrument microplate reader (BioTek, Hercules, CA, FLx800) to analyse the retrieved products of the PCR amplified products. Then, Illumina’s TruSeq Nano DNA LT Library Prep Kit was used to generate indexed libraries for the Illumina MiSeq sequencing. MiSeq Reagent Kit V3 (600 cycles) was used to perform the sequencing by MiSeq sequencing instrument according to the manufacturer’s instructions and used a 2 × 300 paired-end configuration to perform the sequencing.

Statistical analysis

In this project, we used Illumina MiSeq platform to conduct a Paired-end sequencing of duodenal microbial DNA fragments. The raw data that had been sequenced was saved in FASTQ format.

To analyse the raw Paired-end sequencing data, the sliding window method was used to check the quality of the FASTQ format Paired-end sequence: The window size is 10 bp and the step size is 1 bp. It starts to move from the beginning of the 5′. The average quality of the bases in the window was required to be more than Q20, it means that the average sequencing accuracy of base ≥99%. Then cut the sequence from the first window that average quality value lower than Q20, and the length of the cut sequence need to be ≥150 bp, and there are no Ambiguous bases. After that, FLASH (v1.2.7) (Magoc and Salzberg 2011) was be used to pair and connect the qualified Paired-end sequences according to overlapping bases. At the end, according to the Index information of each sample, connected sequence was identified and distributed to the corresponding sample, then we obtained the effective sequence of each sample. Then QIME
software (Quantitative Insights Into Microbial Ecology, v1.8.0) and USEARCH (v5.2.236) was used to check and remove the chimaera sequence produced by PCR. The effective sequences that we got before were used in the final analysis. Using the sequence alignment tool (UCLUST) belong to QIIME software (Edgar 2010) to group sequences into operational taxonomic units (OTUs) at a 97% sequence identity. The most abundant sequence in each OTU was used as the representative sequence of the corresponding OTU. The default parameters in the QIIME software were used to compare the representative sequence of the OTU with the template sequence of the corresponding database. But in actual analysis, not all OTU representative sequences can obtain taxonomic information at the genus or species level (that is ‘Unclassified’ at the corresponding taxonomic level).

Sequences were rarefied before calculating alpha diversity statistics. After that, alpha diversity indices were calculated in QIIME from rarefied sequences using the Chao1 and ACE indexes for richness, and Shannon and Simpson indexes for diversity. Student's t test using IBM SPSS Statistics 24 (IBM Corp., Armonk, NY) was used to perform the statistical analysis and the results are presented as the mean ± standard deviation. A p value <.05 means significant difference between samples. Using the R software to cluster the top 50 genera of abundance and draw the heat map. The R software also be used to construct PLS-DA (Partial Least Squares Discriminant Analysis) model based on species abundance and sample grouping data. And the Metastats (White et al. 2009) statistical algorithm and LEfSe analysis was used to determine the abundance difference of each classification standard between the groups.

Results

Communal OTU analysis

The Venn diagram of common OTU is shown in Figure 1. There were 887 common OTUs between the HS group and the NT group. A total of 254 OTUs from the HS group were acquired, whereas 246 OTUs from the NT group were acquired.

Alpha diversity analysis of duodenal bacteria

The alpha diversity results are presented in Table 2. There are no significant differences in Simpson, Shannon, abundance-based coverage estimators (ACE) and Chao1 richness estimator (Chao1) indexes between NT and HS groups (p > .05).

Beta diversity analysis of duodenal bacteria

Different groups of this experiment have been divided by the oval (5 samples from the HS group and 5 from the NT group). The results show that the NT group and the HS group have a discrepancy on the bacterial community, while D2, D3, D4, D5 samples from both HS and NT group were barely separated from each other (Figure 2).

Taxonomic composition analysis in duodenum

The results show that the NT group have a more abundant bacterial community than HS group in each classification (Table 3), but there are no significant differences of richness between two groups in each classification (p > .05). Then different sample's flora

| Groups | ACE   | Chao1   | Shannon | Simpson |
|--------|-------|---------|---------|---------|
| NT     | 576 ± 162 | 550 ± 153 | 4.64 ± 0.931 | 0.837 ± 0.116 |
| HS     | 559 ± 107  | 534 ± 94.2  | 4.89 ± 0.732  | 0.874 ± 0.067 |

p Value: .849 .842 .644 .551

NT: normal treatment group (21.3 ± 1.2°C); HS: heat stress group (32.5 ± 1.4°C). The relative humidity is 65 ± 5% of two groups.

ACE: abundance-based coverage estimators; Chao1: Chao1 richness estimator.

Figure 2. Partial least squares discriminant analysis (PLS-DA) model of duodenal microbiota from NT and HS broiler groups. NT: normal treatment group (21.3 ± 1.2°C); HS: heat stress treatment group (32.5 ± 1.4°C), the relative humidity is 65 ± 5% of two groups. Different groups of this study have been divided by the different colour ellipses. The blue ellipse is HS group and includes HS.D1-D5 samples, the orange ellipse is NT group and includes NT.D1-D5 samples.
richness (10 samples, five from the HS group and five from the NT group) in two groups were made into Figure 3 by R software. Apart from D3 samples, it can be easily seen that NT group have a more abundant bacterial community than HS group in D1, D2, D4, D5 samples as shown in Figure 3. It can be considered that the difference of D3 samples due to the statistical error.

One taxon of phylum and eight taxon of genus have a significant difference between two groups that had been found ($p < .05$). At the phylum level (Figure 4), broilers in the NT group had a higher relative abundance of Bacteroidetes than those broilers in the HS group ($p < .05$). At the genus level (Figure 5), broilers in the NT group have a higher relative abundance of Cupriavidus, Pseudomonas, Desulfovibrio, Dorea, Janthinobacterium, Leptothrix, Oscillospira and Stenotrophomonas than those broilers in the HS group ($p < .05$).

The relative abundance among taxonomically classified bacteria was further analysed by LEfSe analysis (Figures 6 and 7). We determined the difference of duodenal microbiota on the chicks in the NT group and the HS group. The phyla Bacteroidetes and the corresponding class Flavobacteria and the order of Flavobacteriales and the family of Flavobacteriaceae and the genus of Flavobacterium were significantly decreased in the HS group ($p < .05$). The order of IO25, Xanthomonadales, Rhodocyclales and the corresponding family of those were also significantly decreased in the HS group ($p < .05$). Then the genus richness of Stenotrophomonas, Desulfovibrio, Cupriavidus, and Leptothrix, Dorea were significantly increased in the NT group ($p < .05$).

As shown in Figure 8, the top 50 genera in terms of abundance were clustered and plotted as the Heat map using R software. The populations of Desulfovibrio, Dorea, Blautia, Faecalibacterium, Thermus, and Stenotrophomonas are more abundant in the NT group than the HS group.

Table 3. Effects of heat stress on the number of microbial species at each classification level in duodenum of indigenous yellow-feather broilers.

| Groups   | Phylum        | Class      | Order        | Family        | Genus        | Species |
|----------|---------------|------------|--------------|---------------|--------------|---------|
| NT       | 7.80 ± 1.48   | 14.6 ± 1.82| 24.8 ± 5.63  | 36.4 ± 7.02   | 39.8 ± 12.7  | 16.6 ± 7.92|
| HS       | 7.00 ± 0.707  | 12.8 ± 2.17| 21.0 ± 3.81  | 32.2 ± 7.26   | 33.2 ± 8.64  | 16.2 ± 4.27|
| p Value  | .308          | .193       | .247         | .380          | .364         | .923    |

NT: normal treatment group (21.3 ± 1.2°C); HS: heat stress group (32.5 ± 1.4°C). The relative humidity is 65 ± 5% of two groups.

Discussion

The high temperature causes the drastic changes in intestinal microflora of broiler including the unbalance of intestinal flora, the reduction of beneficial bacteria and the function of cellular processes (Song et al. 2014; He et al. 2018b; Attia et al. 2020). In recent years, the gut microbiome had attracted increasingly attention as an important organ to improve the health of host. In this study, heat stress had attracted increasingly attention as an important organ to improve the health of host. Unfortunately, there were no significant difference in alpha diversity included the Chao1, ACE indexes and Shannon, Simpson indexes in our results, which was in accordance with several studies on caecal microbiota of chicken (Prasai et al. 2016; Liu, Guo et al. 2021).
However, Wang et al. (2018) reported that the Chao1 index was significantly increased after heat stress exposure, and they suggested that heat stress increased the ileal microbiota species richness of broilers. The various results of these studies in response to heat stress could be attributed to the samples from different part of intestine. As we know, the bile produced in the liver can transfer to the duodenum for digestion and detoxication. In the study of Kashihara et al. (2017), the significant reduction of microbiota species richness in the jejunum due to the bile from the duodenum was found, so it can be considered that the high concentration of bile can decrease the increased richness of microbiota by heat stress in the duodenum. Furthermore, the variation of heat stress duration and intensity, broiler strains and diet types could also explain these inconsistent findings (Habashy, Milfort, Fuller et al. 2017; Attia et al. 2020).

According to the taxon comparison results of Metastats and LEfSe analysis, the current study demonstrated heat stress alters the taxonomic composition of duodenum microbiota in broilers, and the significant reduction of Bacteroidetes phylum (Flavobacteria, Flavobacteriales, Flavobacteriaceae, and Flavobacterium) was found in heat-stressed broilers. The similar findings were found in previous studies of Mohd Shaufi et al. (2015) and Shi et al. (2019). Bacteroidetes are capable of metabolising a variety of complex carbohydrates and had been demonstrated that it can typically harbour very broad saccharolytic potential.

**Figure 5.** Taxon comparison of duodenal microbiota between NT and HS broiler groups at the genus level. NT: normal treatment group (21.3 ± 1.2 °C); HS: heat stress group (32.5 ± 1.4 °C), the relative humidity is 65 ± 5% of two groups. *p < .05.

**Figure 6.** Taxonomic column obtained from linear discrimination analysis coupled with effect size (LEfSe) of the most significantly abundant taxa in the duodenal microbiota between NT and HS groups. Only taxa meeting an LDA (Linear discriminant analysis) score significant threshold >2 are shown. NT: normal treatment group (21.3 ± 1.2 °C); HS: heat stress group (32.5 ± 1.4 °C), the relative humidity is 65 ± 5% of two groups.
some species of Bacteroidetes can even target dozens of different complex glycans (Salyers, Vercellotti et al. 1977; Salyers, West et al. 1977; McBride et al. 2003). Previously, Kim and Milner (2007) suggested that Bacteroides could generally produce butyrate, which is an end product of intestinal fermentation. Parkar et al. (2013) also found that Bacteroides played a key role in the synthesis of propionic acid. Butyrate and propionic acid could both protect the gut mucosa from bacterial invasion, and inhibit the pathogens through acidification of the lumen and promote beneficial bacteria occupation of epithelial colonisation sites (Roy et al. 2006). These studies could explain that heat stress induced the reduction of beneficial bacteria by reducing the utilisation efficiency of complex glycans and the production of butyrate and propionic acid via decreasing the colonisation of Bacteroides. However, according to Zhu et al. (2019) and Shi et al. (2019), heat stress also resulted in reduction of Firmicutes, Fusobacteria, and Proteobacteria, and elevation of Anaeroplasma and Lactobacillus phyla in chickens’ faeces and caecal digesta. The variation of gut microbiota in response to heat stress could be associated with the feed formula, growth speed and antioxidant capacity that were demonstrated by Habashy, Milfort, Adomako et al. (2017) and He et al. (2019). The species used in our study (Huaxiang chicken) are highly adaptable to heat stress that could also make an explanation to the inconsistencies among the present study and previous studies.

Moreover, the relative abundance of several putative beneficial genera, including Janthinobacterium, Pseudomonas, Stenotrophomonas, Desulfovibrio, and Oscillospira, were also significantly decreased in heat stressed broiler chickens. It is previously suggested that the Oscillospira genus could utilise the glucuronate and had the ability to produce butyrate, thereby reducing the inflammation of enterocytes in human immature enterocytes (Gophna et al. 2017; Zheng et al. 2020). According to Rubio et al. (2018), the Janthinobacterium could inhibit the growth of pathogenic microorganisms and reduce the morbidity, thus, improving the survival of amphibian. Pseudomonas is well known for its high ability to metabolise fat and degrade various aromatic pollutants in vitro (Mrozik et al. 2004; Goldman et al. 2010). Regarding the Stenotrophomonas, Berg and Martinez (2020) suggested that a lower relative abundance of...
Stenotrophomonas might be due to the absence of heat shock genes and/or the up-regulation of several genes involved in a suicide mechanism during heat stress. According to Ryan et al. (2009), they demonstrated that Stenotrophomonas perform the biocontrol of oomycete, fungal and bacterial pathogens, and also have an extraordinarily high hydrolytic potential of fat. Surprisingly, Desulfovibrio genus are capable of using hydrogen that released during fermentation by phyla Bacteroidetes, and promote their metabolism in chickens’ faeces (Vignais and Billoud 2007; Videnska et al. 2014). Hence, the current results indicated that heat stress might also reduce the metabolic capacity of Bacteroides phylum, promote inflammation, and increase pathogen invasion by decreasing the colonisation of beneficial bacteria genus. However, further researches are necessary to confirm the findings and to illustrate the underlying mechanism.

Conclusion

In summary, this study demonstrated that high temperature environment exposure (4 weeks, 32.5 ± 1.4 °C 10 h/day) altered the microbiota composition of indigenous broilers. Particularly, the phyla Bacteroidetes and the genera Cupriavidus, Leptothrix, Janthinobacterium, Pseudomonas, Stenotrophomonas, Desulfovibrio, Oscillospira, and Dorea were decreased by heat stress exposure. The present findings provided a mechanism foundation of regulating the gut microbiota as anti-heat stress strategy when raising indigenous yellow-feather broilers in tropical and/or subtropical regions.

Disclosure statement

The authors declare they have no conflict of interest.

Ethical approval

The animal study in the present experiment was reviewed and approved by Animal Care Committee, Guangdong Ocean University (Zhanjiang, Guangdong, China).

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Data availability statement

All data are included and reported in the article.

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