

Research Note: Increase of bad bacteria and decrease of good bacteria in the gut of layers with vs. without hepatic steatosis

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ABSTRACT Fatty liver hemorrhagic syndrome (**FLHS**), usually occurring in hens in the late laying period, can lower egg yield and even cause death. Similar to nonalcoholic fatty liver disease in humans, FLHS may begin with simple hepatic steatosis. It is known that gut microbiota is important to the development of nonalcoholic fatty liver disease, but the relationship between FLHS and gut bacteria remains unclear. In this study, bacteria compositions in the ileum and cecum were determined by 16S rDNA sequencing analysis in the groups of hens with vs. without hepatic steatosis (average liver fat contents were 0.34 mmol/g protein or 2.6% vs. 0.84 mmol/g protein or 6.0%). These hens were in the 2 tails of the liver fat content distribution with each tail accounting for 10% of the test population containing 90 66-wk-old healthy Rhode Island Red laying hens raised under routine feeding regimen. The results showed that liver weight but not the weights of the body, heart and abdominal fat were significantly different between the groups. Moreover, bacterial diversity was not

significantly different between the groups, but bacterial diversity in the cecum was higher than that in the ileum. Proteobacteria was the most dominant phylum in the ileum, whereas Proteobacteria, Firmicutes, and Bacteroidetes were the most dominant phyla in the cecum. Some bacteria were common to the ileum and cecum, but some were unique. Furthermore, some bacteria were significantly different in abundance between the groups, that is the group without hepatic steatosis had more bacteria inhibiting host energy absorption or benefiting intestinal health, whereas the group with hepatic steatosis had more conditionally pathogenic or harmful bacteria. In conclusion, hepatic steatosis in laying hens is associated with intestinal bacterial composition (bacterial abundance) but not bacterial diversity. The identified common and unique bacteria are related to the functional characteristics of the ileum and cecum. The decrease of beneficial bacteria and the increase of harmful bacteria in the intestine of laying hens may increase FLHS incidence by promoting hepatic steatosis.

Key words: fatty liver hemorrhagic syndrome, chicken, gut microbiota, metabolic disease

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INTRODUCTION

The avian egg is one of the important sources of protein. It is delicious, balanced in nutrient content, and very popular with consumers. In recent years, the global annual production of eggs has exceeded 70 million tons, and the annual stock of laying hens is about 3.85 billion. However, the production performance of laying hens in the late period of laying, especially those raised in cage, sometimes is affected by fatty liver hemorrhage

syndrome (**FLHS**), which is caused by a disorder in lipid metabolism (i.e., an imbalance between synthesis and transportation/oxidation of fatty acids) and characterized by excessive deposition of fat in the liver. Raising laying hens in cages is very common in the industry, with more than 90% of laying hens raised in cage in the 3 major egg producing countries (China, Japan, and the United States). Fatty liver hemorrhage syndrome can lower laying performance and egg quality and in severe cases can cause death, which results in a great economic loss to the industry. However, the pathogenesis of FLHS is still unclear.

In humans and rodents, nonalcoholic fatty liver disease (**NAFLD**) is initiated with simple steatosis. The criterion for hepatic steatosis is that liver fat weight exceeds 5% of liver weight ([Beaudry and Devries, 2019](#)). As both NAFLD and FLHS are characterized by hepatic steatosis, FLHS may be similarly initiated with simple

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steatosis. Recent evidence indicates that gut microbes play an important role in the development of NAFLD. For example, different gut microbial composition can affect the response of mice to high-fat diets and determine the degree of hepatic steatosis other than obesity (Le Roy et al., 2013). Compared with the studies in humans and mice, studies in layers on the relationship between NAFLD and gut microbiota are rare. The findings from the limited studies in laying hens indicate that high-fat diets can induce nonalcoholic steatohepatitis and dysbiosis of gut bacteria and that the change in the abundances of *Bacteroides* and *Lachnospiraceae* genera is closely associated with the degree of liver fibrosis and the severity of nonalcoholic steatohepatitis (Hamid et al., 2019). Based on these findings, we proposed a hypothesis that hepatic steatosis in laying hens was associated with the change of intestinal microbiota composition. To test it, bacterial composition in the ileum and cecum between laying hens with and without hepatic steatosis was determined by 16S rDNA sequencing analysis. The results may provide a foundation for further research on the pathogenesis of FLHS.

MATERIALS AND METHODS

Experimental Animals and Sample Collection

A total of 90 healthy 66-wk-old Rhode Island Red laying hens were randomly selected from a population raised under routine feeding regimen and routine husbandry managements in Jiang Su Bei Nong Da Agricultural Animal Husbandry Technology Co., Ltd., Taizhou, China. The hens were in cages with free access to water and feed. Their average BW at the age of 66 wk was 2.022 kg, and their average laying rate was 82.5%. The weights of the liver, heart, and abdominal fat were measured after sacrifice. The liver samples (middle part of the right lobe) and intestinal contents (about 0.5–2 g) were collected from each bird and stored at -70°C . Based on liver fat content, the individuals in the right and left tails of the liver fat content distribution were grouped as the birds with and without hepatic steatosis (i.e., group A and B) ($n = 9$), respectively. The intestinal contents of the groups were subjected to 16S rDNA sequencing analysis. All animal protocols were approved by the Animal Care and Use Committee of Yangzhou University (the certificate number authorized by IACUC is SYXK(Su)2016-0020). The animals tested in this study were treated as humanely as possible and every effort was made to minimize the sufferings of the animals during this study.

Measurement of Triglyceride Content in the Liver

The triglyceride (TG) level in the liver was determined with glycerol phosphate oxidase-phenol aminophenazone method using a commercial kit (Assay kit A110-1, Nanjing Jiancheng Biotechnology Co., Ltd., Nanjing,

China) as per the manufacturer's instructions. The protein concentration in the homogenate of the liver sample used for measurement of TG content was also determined with Assay kit a045-2 (Nanjing Jiancheng Biotechnology Co., Ltd.) as per the manufacturer's instructions. The TG content in the liver was finally normalized to the protein concentration (mmol TG per g of protein) or to the liver weight.

16S rDNA Sequencing Analysis

The 16S rDNA sequencing analysis was conducted by Gene Pioneer Biotechnologies, Nanjing, China. The method was briefly described as follows: DNA in the intestinal content of the ileum and cecum was isolated with HiPure Soil DNA Kit (D3142; Magen Biotechnology Co., Ltd., Shanghai, China) as per the manufacturer's instructions. The qualified DNA samples were used for 16S rDNA sequencing analysis. The PCR primers for 16S rDNA gene (only the fourth and fifth variable regions, namely V4 and V5 regions) were 5'-GTGCCAGC(A/C)GCCGCGG-3', 5'-CCGTCAATTC (A/C)TTT(A/G)AGTTT-3'. After sequencing on Hiseq2500 PE250, the raw reads were spliced and filtered; only the qualified clean reads were clustered by operational taxonomic units as per the principle of 97% similarity. By comparing the sequence of operational taxonomic units with SILVA rDNA database (<http://www.arb-silva.de/>), the species classification information corresponding to each operational taxonomic unit was retrieved. The alpha diversity index was calculated by Qiime 1.9.1 software, and the difference in alpha diversity was analyzed with 2-way ANOVA using GLM procedure provided by IBM SPSS Statistics 25.0 software. Finally, the composition and distribution of gut bacteria were compared between the groups.

Statistical Analysis

The data were analyzed with 2-way ANOVA using GLM procedure or *t* test provided by IBM SPSS Statistics 25.0 software to determine statistical significance. The criteria for statistical significance was set as $P < 0.05$. The values were expressed as the means \pm SE.

RESULTS AND DISCUSSION

Fatty liver hemorrhagic syndrome in laying hens is characterized by excessive accumulation of fat in the liver, and simple steatosis may be its initial stage. As intestinal bacteria play an important role in the formation of human and rodent NAFLD, we speculate that intestinal bacteria also contribute to the development of FLHS in laying hens. In this study, the relationship between gut microbiota and liver fat content was investigated in laying hens under routine feeding regimen. Data showed that the average liver fat contents were 0.34 mmol/g protein or 2.6% for group A vs. 0.84 mmol/g protein or 6.0% for group B ($n = 9$). There were no significant differences in BW, heart weight, and

abdominal fat weight between the groups, but liver weight (38.04 ± 6.76) and the ratio of liver weight to BW (1.98 ± 0.41) in group A was significantly greater than those in group B (28.08 ± 5.12 , 1.43 ± 0.21).

In regard to gut microbiota, there was no significant difference in the diversity of intestinal microbes for the ileum or cecum between the groups, indicating that liver fat content is not associated with the diversity of gut microbiota in laying hens. This finding is inconsistent with the decrease in the diversity of gut microbiota shown in previous studies on fatty liver in humans, geese, and other animals. This discrepancy may be due to the differences in diet composition, raising condition (routine feeding regimen vs. overeating), and between

pathologic and physiological states or due to the special physiological state of laying hens during the laying period. However, the alpha diversity index of gut microbiota was significantly different between the ileum and the cecum, with bacterial diversity in the cecum higher than that in the ileum, regardless of liver TG content. The difference in the bacterial diversity between the ileum and cecum may be owing to their different positions and different internal environments in the digestive tract as well as their different functions.

In regard to the dominant phyla and genera of bacteria in the ileum (Figure 1A) and cecum (Figure 1B), data showed that Proteobacteria phylum was absolutely dominant in the ileum, while Bacteroidetes, Firmicutes,

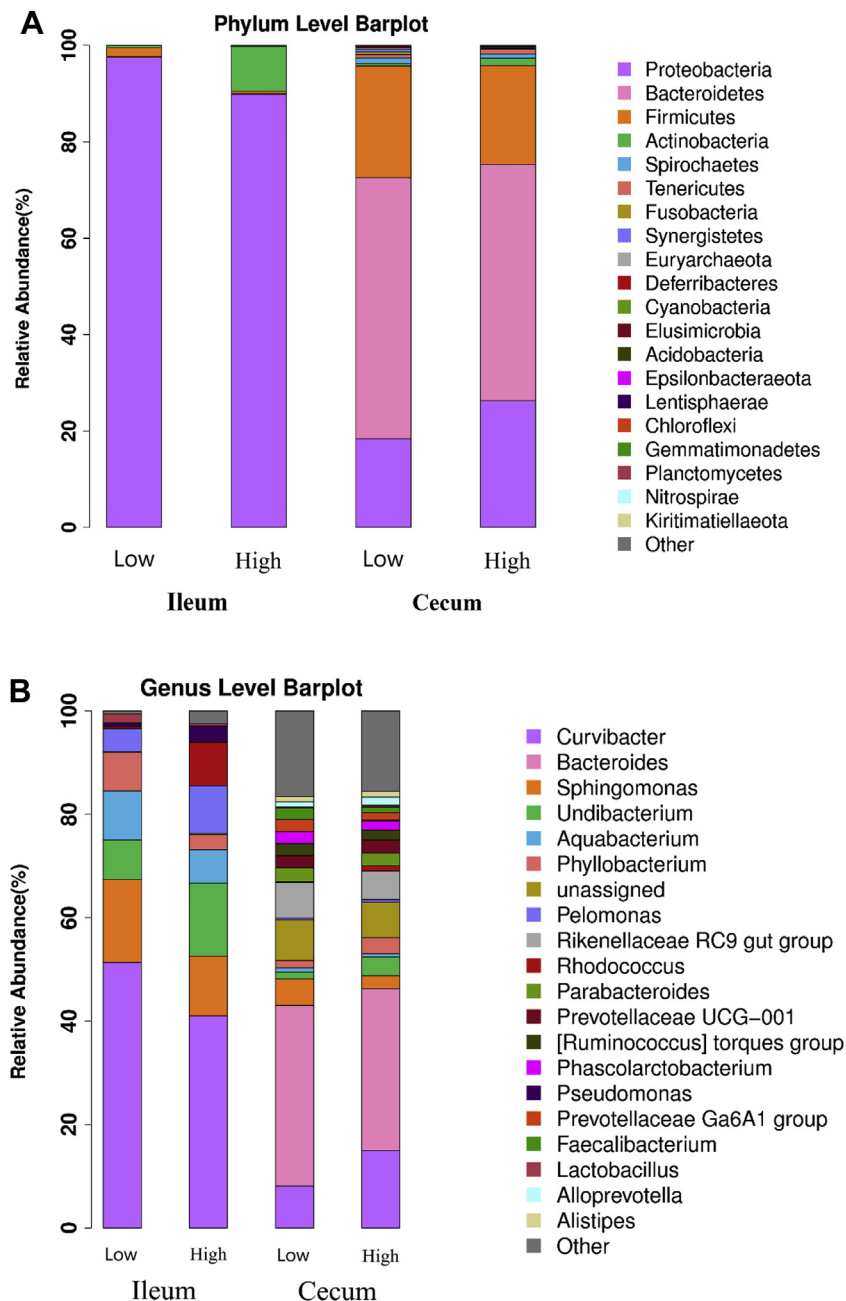


Figure 1. Distribution of bacteria in the ileum and cecum of laying hens with (High) and without (Low) hepatic steatosis. (A) The relative abundances of bacteria at the phylum level in the ileum and cecum of laying hens with and without hepatic steatosis. (B) The relative abundances of bacteria at the genus level in the ileum and cecum of laying hens with and without hepatic steatosis. $n = 9$.

and Proteobacteria phyla were dominant in the cecum. Proteobacteria phylum can degrade the nutrients in the diet (e.g., *Pseudomonas* can degrade protein and fat) and provide suitable environmental support for symbiotic bacteria. Changes in the abundance of Proteobacteria phylum are regarded as one of the signs of gut microbiota dysbiosis and the key to determine intestinal health of animals (Shin et al., 2015). Bacteroidetes phylum can use the degraded energy materials to inhibit host nutritional absorption and provide nutrition for other intestinal bacteria. Firmicutes phylum can produce monosaccharide and short-chain fatty acids (SCFAs) by degrading dietary fiber. Monosaccharide and SCFA are used by intestinal bacteria and the host, respectively. SCFA also contribute to the maintenance of intestinal structural integrity and barrier function. For the dominant genera of bacteria, some dominant genera of bacteria were common to the ileum and cecum, and some were unique (Figure 1B). Interestingly, among the dominant genera of bacteria, previous studies have shown that some are associated with NAFLD. For example, high-fat diets can significantly reduce the number of *Phascolarctobacterium* genus in the intestinal tract, which makes the rats more susceptible to NAFLD (Panasevich et al., 2016). *Parabacteroides* genus cultured in vitro has the ability to transform bile acid and produce succinic acid, while supplementation of succinic acid to diet can activate intestinal gluconeogenesis, thus reducing hyperglycemia in ob/ob mice. *Faecalibacterium* genus has certain anti-inflammatory effect, which may alleviate inflammation in NAFLD (Iino et al., 2019). In one word, the dominant bacteria do not only provide important support for maintaining the structural stability of gut microbiota and host intestinal health but also protect animals from some metabolic disorders, including NAFLD.

In regard to the bacteria with abundance different between the groups, data showed that the abundance of *Bacteroides* phylum in the ileum was lower in the group without hepatic steatosis (0.097%) than the group with hepatic steatosis (0.272%), whereas the abundance of Firmicutes phylum was higher in the group without hepatic steatosis (1.83%) than the group with hepatic steatosis (0.505%) (Table 1). So, the ratio of *Bacteroides* to Firmicutes was obviously lower in the group without hepatic steatosis than the group with hepatic steatosis, which is inconsistent with previous studies on fatty liver in humans, geese, and other animals. However, the abundances of both *Lactobacillus* genus of Firmicutes phylum and *Bacteroides* genus of Bacteroidetes phylum, which are beneficial to intestinal health (Heeney et al., 2018), were significantly higher in the group without hepatic steatosis than the group with hepatic steatosis. Moreover, the abundance of Proteobacteria phylum in the ileum was significantly higher in the group without hepatic steatosis than the group with hepatic steatosis, which seemed to be inconsistent with other study reports (i.e., the increase in the abundance of Proteobacteria phylum is often associated with microbial dysbiosis). Surprisingly, the abundances of *Undibacterium*, *Pseudomonas*, and *Pelomonas*

Table 1. Differential gut bacteria in the ileum and cecum of laying hens with (high) and without (low) hepatic steatosis.

Bacteria	Low	High
Ileum		
Proteobacteria	97.7 ± 0.780^a	89.8 ± 4.88^b
<i>Aquabacterium</i>	9.05 ± 2.2 ^a	5.84 ± 2.3 ^b
<i>Curvibacter</i>	49.8 ± 4.9 ^a	38.9 ± 6.4 ^b
<i>Pelomonas</i>	4.24 ± 1.2 ^a	8.19 ± 3.4 ^b
<i>Phyllobacterium</i>	7.23 ± 2.2 ^a	2.93 ± 2.7 ^b
<i>Pseudomonas</i>	0.671 ± 0.3 ^a	2.78 ± 1.5 ^b
<i>Sphingomonas</i>	15.6 ± 2.9 ^a	11.2 ± 4.7 ^b
<i>Undibacterium</i>	7.32 ± 1.8 ^a	13.3 ± 2.7 ^b
Actinobacteria	0.430 ± 0.312^a	0.925 ± 4.92^b
<i>Rhodococcus</i>	0.396 ± 0.3 ^a	8.06 ± 0.50 ^b
Firmicutes	1.83 ± 0.820	0.505 ± 0.252
<i>Lactobacillus</i>	0.975 ± 0.4 ^a	0.371 ± 0.2 ^b
Bacteroidetes	0.097 ± 0.034	0.272 ± 0.107
<i>Bacteroides</i>	0.024 ± 0.01 ^a	0.014 ± 0.01 ^b
Cecum		
Firmicutes	20.7 ± 2.15	18.5 ± 1.82
<i>Angelakisella</i>	0.007 ± 0.002 ^a	0.001 ± 0.001 ^b
<i>Flavonifractor</i>	0.143 ± 0.029 ^a	0.067 ± 0.008 ^b
<i>Megasphaera</i>	0.086 ± 0.031 ^a	0.399 ± 0.058 ^b
<i>Ruminococcaceae UCG-005</i>	0.725 ± 0.119 ^a	0.388 ± 0.074 ^b
<i>Ruminococcaceae NK4A214</i>	0.461 ± 0.089 ^a	0.232 ± 0.042 ^b
<i>Ruminiclostridium 9</i>	0.865 ± 0.066 ^a	0.634 ± 0.085 ^b
Fusobacteria	0.514 ± 0.134^a	0.172 ± 0.056^b
<i>Fusobacterium</i>	0.514 ± 0.134 ^a	0.172 ± 0.056 ^b
<i>Novosphingobium</i>	0.006 ± 0.002 ^a	0.018 ± 0.006 ^b
Synergistetes	0.468 ± 0.100^a	0.162 ± 0.033^b
<i>Butyrivimonas</i>	0.020 ± 0.006 ^a	0.005 ± 0.005 ^b
Euryarchaeota	0.306 ± 0.075^a	0.151 ± 0.030^b
<i>Methanocorpusculum</i>	0.170 ± 0.035 ^a	0.068 ± 0.022 ^b

All data are expressed as the mean ± SE. The values are the relative percentage (%) indicating the relative abundance of a specified phylum (Bold) or genus (regular) of bacteria in all intestinal bacteria. The values with different superscript letters in the same row indicate that the means are significantly different between the group with and without hepatic steatosis ($P < 0.05$). n = 9.

genera were significantly lower in the group without hepatic steatosis than the group with hepatic steatosis, whereas the abundances of *Curvibacter*, *Sphingomonas*, *Phyllobacterium*, and *Aquabacterium* genera were significantly higher in the group without hepatic steatosis than the group with hepatic steatosis. It is known that *Sphingomonas* genus can synthesize sphingosine with nutrients, and sphingosine can form sphingomyelin with derivatives of fatty acids, thus reducing fat deposition in the liver. *Phyllobacterium* genus is a chemoheterotrophic bacterium, which can compete with the host for nutrient absorption and utilization. On the contrary, *Pseudomonas* genus is a conditional pathogen, which can cause a variety of infections when host immunity is low (Meynet et al., 2018). In addition, the abundance of Actinobacteria phylum (e.g., *Rhodococcus*) in the ileum was significantly lower in the group without hepatic steatosis than the group with hepatic steatosis. *Rhodococcus* genus is also a conditional pathogen, which has strong infection ability under specific conditions. It has been reported that *Rhodococcus* genus is related to the case of human immune dysfunction (Kim et al., 2018). In summary, there were more bacteria that can inhibit host energy absorption and benefit intestinal health in the ileum of the group without hepatic steatosis, whereas there were more conditional pathogens or harmful bacteria in the ileum of the group with hepatic steatosis.

In the cecum, there was almost no difference in the abundance of Firmicutes and Bacteroidetes phyla between the groups. However, the abundances of Fusobacteria, Synergistetes, and Euryarchaeota phyla in the group with hepatic steatosis were significantly lower than that in the group without hepatic steatosis. Moreover, the abundances of *Methanocorpusculum*, *Ruminococcaceae* UCG-005, *Ruminococcaceae* NK4A214 group, *Flavonifractor*, *Ruminiclostridium* 9 and *Angelikisella*, *Butyricimonas*, and *Fusobacterium* genera were significantly lower in the group with hepatic steatosis than those in the group without hepatic steatosis, whereas the abundances of *Megasphaera* and *Novosphingobium* genera were significantly higher in the group with hepatic steatosis than those in the group without hepatic steatosis. Previous studies have shown that the reduction in the abundance of *Ruminococcaceae* genus is significantly associated with obesity and the deterioration of fatty liver disease in humans. *Ruminococcaceae* genus is the main member producing SCFA and plays an important role in regulating intestinal pH, maintaining immune homeostasis, and improving intestinal structure and health (Ratajczak et al., 2019). On the other hand, *Fusobacterium* and *Synergistetes* genera are conditional pathogens, which may increase the risk of intestinal diseases in host animals. Therefore, the change of microbiota composition in the cecum is quite similar to that in the ileum.

In conclusion, liver fat content was not associated with the diversity of intestinal microbes, and bacterial diversity in the cecum was higher than that in the ileum. There were some bacteria unique to the ileum and cecum, which may be related to different functional characteristics of the intestinal segments. Compared with laying hens without hepatic steatosis, those with hepatic steatosis had less beneficial bacteria and more conditional pathogens or harmful bacteria in the intestine.

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