


Caecal microbiota could effectively increase chicken growth performance by regulating fat metabolism

Xiaolong Zhang,^{1,†} Yafang Hu,^{1,†}
Abdur Rahman Ansari,^{1,2}  Muhammad Akhtar,¹
Yan Chen,¹ Ranran Cheng,¹ Lei Cui,¹
Abdallah A. Nafady,¹ Abdelmotaleb A. Elokil,^{1,3}
El-Sayed M. Abdel-Kafy⁴ and Huazhen Liu^{1,*}

¹Department of Basic Veterinary Medicine, College of Animal Science and Veterinary Medicine, Huazhong Agricultural University, Wuhan, Hubei 430070, China.

²Section of Anatomy and Histology, Department of Basic Sciences, College of Veterinary and Animal Sciences (CVAS) Jhang, University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan.

³Department of Animal Production, Faculty of Agriculture, Benha University, Moshtohor, 13736, Egypt.

⁴Animal Production Research Institute (APRI), Agricultural Research Center (ARC), Ministry of Agriculture, Giza, Egypt.

abundantly ($P < 0.05$). The results of H & E, qPCR, IHC, WB and blood analysis suggested significantly different fat metabolism level in serum, liver, abdominal adipose, breast and leg muscles between high and low body weight chickens. Spearman correlation analysis revealed that fat metabolism positively correlated with the relative abundance of *Microbacterium* and *Sphingomonas* while negatively correlated with the abundance of *Slackia*. Furthermore, faecal microbiota transplantation was performed, which verified that transferring faecal microbiota from adult chickens with high body weight into one-day-old chickens improved growth performance and fat metabolism in liver by remodelling the gut microbiota. Overall, these results suggested that gut microbiota could affect chicken growth performance by regulating fat metabolism.

Summary

It has been established that gut microbiota influences chicken growth performance and fat metabolism. However, whether gut microbiota affects chicken growth performance by regulating fat metabolism remains unclear. Therefore, seven-week-old chickens with high or low body weight were used in the present study. There were significant differences in body weight, breast and leg muscle indices, and cross-sectional area of muscle cells, suggesting different growth performance. The relative abundance of gut microbiota in the caecal contents at the genus level was compared by 16S rRNA gene sequencing. The results of LEfSe indicated that high body weight chickens contained *Microbacterium* and *Sphingomonas* more abundantly ($P < 0.05$). In contrast, low body weight chickens contained *Slackia* more

Introduction

In animal production, antibiotics have been used as feed additives to enhance growth performance. Poultry industry is one of the largest food industries worldwide, and chicken is the common species reared at farms for meat production (Agyare *et al.*, 2018). Many countries are using a range of antibiotics for chicken production (Sahoo *et al.*, 2010; Landers *et al.*, 2012). However, long-term use of antibiotics can produce antibiotic-resistant zoonotic pathogens and cause antibiotic residues in food (Allen *et al.*, 2014; Pamer, 2016). In addition, poorly absorbed antibiotics are excreted unchanged in faeces and urine, and dispersed in soil when manure is used as a fertilizer, resulting in an antibiotic pollution in the environment (Joy *et al.*, 2014). Consequently, the use of antibiotics as a growth promoter has been banned in Sweden since 1986, in the European Union and other countries (Case well *et al.*, 2003; Hoese *et al.*, 2009; Maron *et al.*, 2013). Several countries have withdrawn the use of some antibiotics, yet among all produced antibiotics, about 60% is estimated to be used in livestock production, especially in poultry and pigs (Van Boeckel *et al.*, 2014; Van Boeckel *et al.*, 2015). Therefore, the development of alternatives to antibiotic growth promoters (AGPs) is an important strategy in animal production and food safety.

The chicken gastrointestinal tract harbours a complex consortium of microbial communities, with the highest bacterial diversity in the caecum (Bjerrum *et al.*, 2006;

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*For correspondence. E-mail lhaz219@mail.hzau.edu.cn; Tel. (+86) 27 87286970; Fax (+86) 27 87280408.

[†]Authors contributed equally as first co-authors
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Wei *et al.*, 2013; Awad *et al.*, 2016; Saxena *et al.*, 2016; Pandit *et al.*, 2018). Multiple studies in chickens have established the importance of the gut microbiota, especially the caecal microbiota, in improving feed digestion, nutrient absorption and growth performance (Stanley *et al.*, 2014; Stanley *et al.*, 2016; Yan *et al.*, 2017). Notably, alteration of the gut microbiota using faecal microbiota transplantation or probiotic supplementation as an alternative to antibiotics has also been reported to improve chicken growth performance, indicating that the gut microbiota is an essential resource for developing natural growth promoters (Angelakis, 2017; Siegerstetter *et al.*, 2018; Videvall *et al.*, 2019; Yadav and Jha, 2019).

Fat metabolism is an important and complex biochemical reaction, including the processes of digestion, absorption, synthesis and catabolism (D'Aquila *et al.*, 2016). Balanced fat metabolism can improve host growth performance and meat quality, yet imbalanced fat metabolism results in obesity and disease. Accumulating investigations focussing on the relationship between gut microbiota and obesity suggested that the gut microbiota is an important environmental factor affecting energy harvest from the diet and energy storage in mammals (Bäckhed *et al.*, 2004; Turnbaugh *et al.*, 2006; Wang *et al.*, 2017). It has been reported that 12 *Lactobacillus* strains reduced triglycerides, abdominal fat deposition and total serum cholesterol in broilers (Kalavathy *et al.*, 2003; Wang *et al.*, 2017). Independent to the host genetic factors, duodenal as well as caecal microbiota of chicken plays a critical role in fat deposition (Wen *et al.*, 2019). Abundantly observed beneficial bacteria, that is *Bacteroides* and *Lactobacillus* in the chicken gut, were associated with high body weight gain, low abdominal fat, high breast muscle yield and increased growth performance (Zheng *et al.*, 2019). Whether the gut microbiota affects chicken growth performance by regulating fat metabolism becomes an interesting question.

To tackle this question, chickens from the same group with different growth performance were used in the present study to compare fat metabolism levels and gut microbial communities. The correlation between fat metabolism and gut microbiota was analysed using Spearman correlation analysis. Besides, transferring faecal microbiota from adult chickens with high body weight into one-day-old chicks was performed to verify whether chicken gut microbiota affected growth performance by regulating fat metabolism.

Results

Difference in growth performance

Seven-week-old chickens from the same group with significantly different body weight ($P = 0.001$) (Fig. 1A) were selected. There were significant differences in

breast muscle index ($P = 0.014$) (Fig. 1B) and leg muscle index ($P = 0.0489$) (Fig. 1C). Haematoxylin and eosin (H & E) staining results indicated that the average cross-sectional areas of breast muscle cells ($P = 0.0105$) (Fig. 1D) and leg muscle cells ($P = 0.0304$) (Fig. 1E) were significantly larger in high than in low body weight chickens.

Differences in caecal microbiota

The chickens' caecal microbiota was analysed using 16S rRNA gene sequencing. A total of 1 341 412 high-quality reads were obtained from 20 samples (an average of 67 070 reads per sample), which were clustered into 748 operational taxonomic units (OTUs) at 97% sequence similarity. The alpha diversity results exhibited no significant differences in microbial diversity in caecal contents between high and low body weight chickens (Fig. 2A-B). However, beta diversity showed some separation between high and low body weight groups (Fig. 2C). At the phylum level, Firmicutes, Bacteroidetes and Proteobacteria were observed as the most abundant phyla. The relative abundance of Firmicutes was lower, while of Bacteroidetes was higher in high body weight chickens (Fig. 2D). The ratio of Firmicutes to Bacteroidetes was significantly lower in high body weight chickens (Fig. 2E). At the genus level, *Bacteroides* were observed as the dominant genus in caecal contents (Fig. 2F). Linear discriminant analysis effect size (LEfSe) analysis revealed that the relative abundances of *Faecalibacterium*, *Microbacterium*, *Slackia*, and *Sphingomonas* were significantly different in high and low body weight chickens (Fig. 2G).

Differences in liver fat metabolism

H & E staining results indicated more vacuolar fat in the livers of high body weight chickens (Fig. 3A). The relative mRNA expression of fat synthesis-related genes including cytochrome P450 2C45 (CYP2C45) ($P = 0.0373$), fatty acid desaturase 1 (FADS1) ($P = 0.0256$) and acyl-CoA synthetase long chain family member 1 (ACSL1) ($P = 0.0164$) (Fig. 3B) and the fat catabolism-related genes fasting-induced adipose factor (fiat) ($P = 0.0384$), peroxisome proliferator-activated receptor alpha (PPAR α) ($P = 0.0234$) and carnitine palmitoyl transferase I (CPT-1) ($P = 0.0319$) (Fig. 3C) were significantly higher in the livers of high body weight chickens than in low body weight chickens. The relative mRNA expression of fat synthesis-related genes acetyl CoA carboxylase (ACC) and fatty acid synthase (FAS) were higher in high body weight chickens as compared to low body weight chickens, but not significantly. The relative mRNA expression of fat transport-related gene apolipoprotein A-I (ApoAI) was

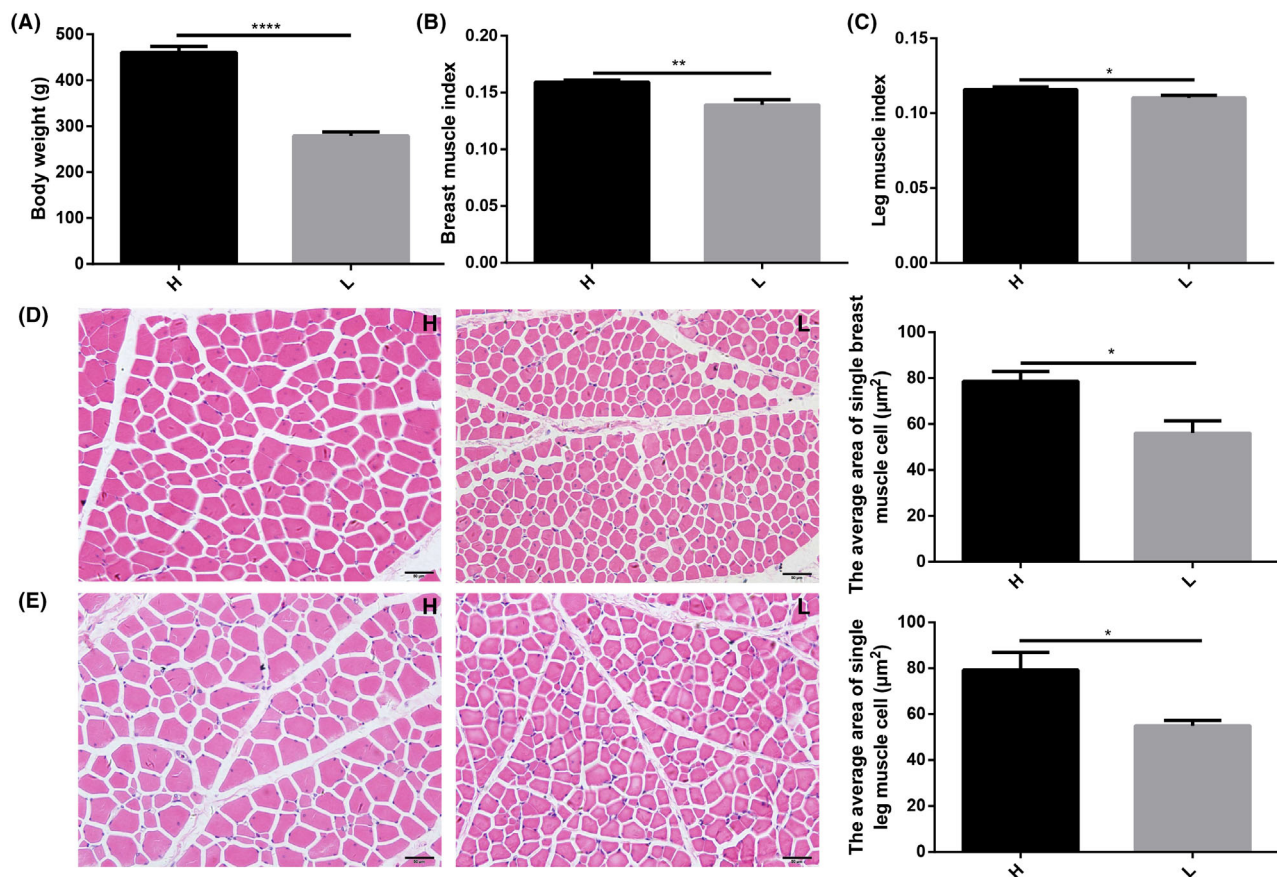


Fig. 1. Different growth performance between high and low body weight chickens. Chickens (Turpan cockfighting × White Leghorn) at 7th week of age were selected to analyse the growth performance of high and low body weight chickens ($n = 10$). Each group contained five males and five females. (A) Body weight. (B) Breast muscle index. (C) Leg muscle index. (D) Section of breast muscle with H & E staining and the comparison of single cell's cross-sectional area. (E) Section of leg muscle with H & E staining and the comparison of single cell's cross-sectional area. H represented high body weight chickens and L represented low body weight chickens. Scale bars = 50 μm . All data were presented as the means \pm SEM. P values were calculated using Student's t -test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

significantly higher in the liver of high body weight chickens than in low body weight chickens ($P = 0.0166$) (Fig. 3D). Immunohistochemistry (IHC) staining indicated that phospho-AMP-activated protein kinase (P-AMPK) was distributed in the cytoplasm and nucleus of hepatocytes in liver tissue (Fig. 3E), and the protein expression of P-AMPK was significantly higher in high body weight chickens than that in low body weight chickens ($P = 0.0095$) (Fig. 3F). Western blot results also revealed a higher protein expression of P-AMPK in high body weight chickens (P-AMPK/GAPDH, $P = 0.0045$; P-AMPK/AMPK, $P = 0.0008$) (Fig. 3G–I).

Differences in fat metabolism in blood and abdominal adipose

In abdominal adipose, H & E staining results showed that adipocytes were vacuolar, and the number of adipocytes in abdominal adipose tissue was significantly

higher in high body weight chickens rather than in low body weight chickens ($P = 0.0056$) (Fig. 4A). The average diameter of adipocytes was significantly smaller in high than in low body weight chickens ($P = 0.0063$) (Fig. 4B). In serum of high body weight chickens, the concentration of high-density lipoprotein cholesterol (HDL-C) was significantly ($P = 0.0436$) higher, while the concentration of low-density lipoprotein cholesterol ($P = 0.0273$) (LDL-C) was significantly lower compared with low body weight chickens (Fig. 4C). There were no significant differences in the concentrations of triglyceride (TG) or total cholesterol (TC) between high and low body weight chickens. qPCR results showed that the relative mRNA expression levels of ACC and FAS were lower in high body weight chickens than in low body weight chickens, but there were no significant differences (Fig. 4D). The relative mRNA expression levels of adipocyte differentiation-related genes, including sterol regulatory element-binding protein 1 ($P = 0.0128$)

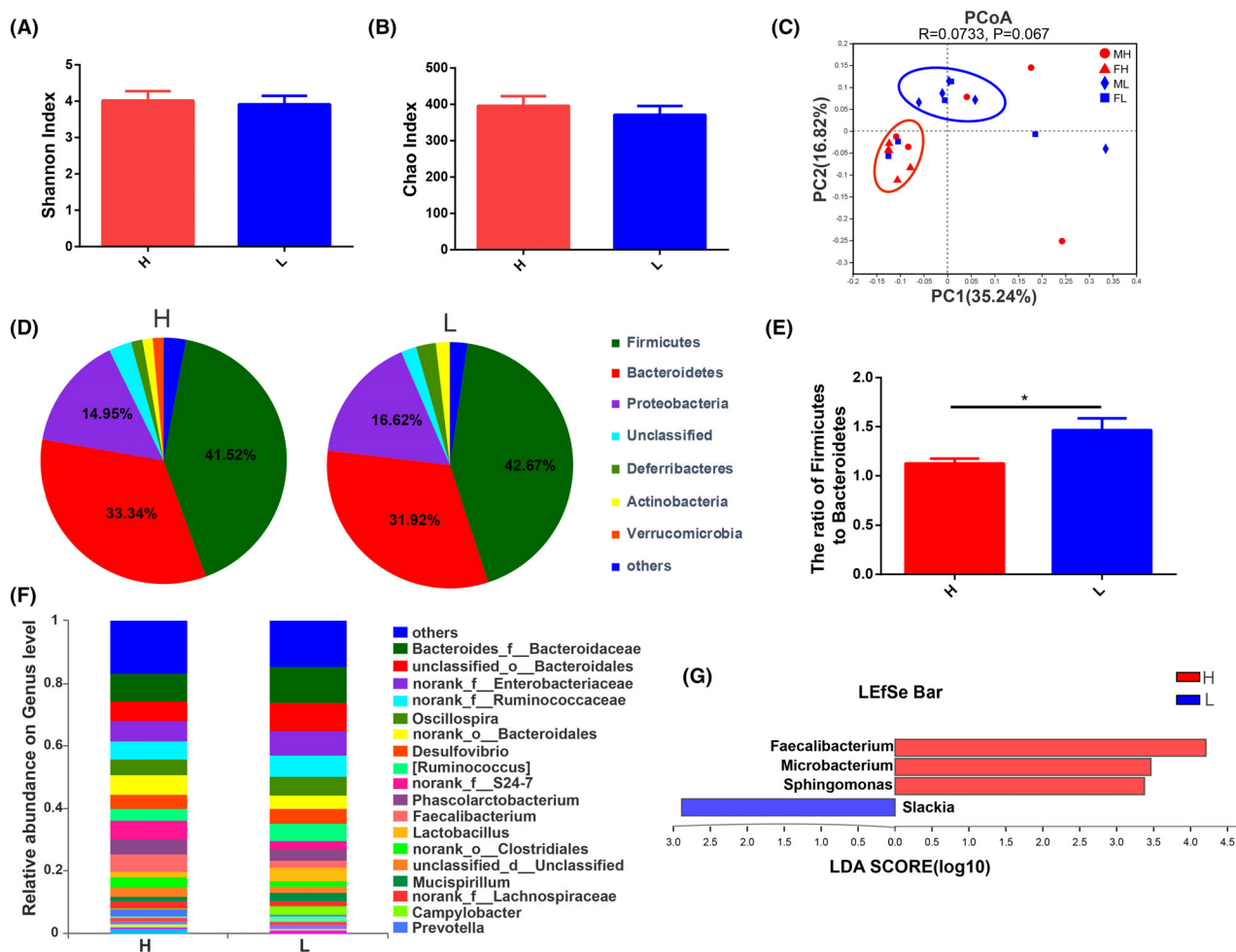


Fig. 2. Differences in caecal microbiota between high and low body weight chickens.

- A. Chickens (Turpan cockfighting × White Leghorn) at 7th week of age were sacrificed to perform 16S rRNA sequence of caecal microbiota ($n = 10$). Each group contained five male and five female chickens. Microbial community diversity (measured by the Shannon index).
- B. Microbial community abundance (measured by the Chao index).
- C. Beta diversity of high and low body weight chickens. MH: male with high body weight, FH: female with high body weight, ML: male with low body weight, FL: female with low body weight.
- D. The composition of microbial communities at the phylum level.
- E. The ratio of Firmicutes to Bacteroidetes.
- F. The composition of microbial communities at the genus level.
- G. LDA score for LEfSe analysis (LEfSe was done at LDA score of >2).

(SREBP1) and adiponectin ($P = 0.0401$), were significantly lower in high body weight chickens. The adipocyte protein 2 (AP2) and PPARG were also lower in high body weight chickens, but there were no significant differences (Fig. 4E).

Differences in fat metabolism in breast and leg muscles

There was no significant difference in the relative expression of ACC in breast muscle and leg muscle between high and low body weight chickens (Fig. 5A). The relative mRNA expression of the fat transport-related gene (A-FABP) was much lower ($P = 0.0001$) in the breast

muscle of high body weight chickens (Fig. 5B), while the relative mRNA expression of the fat catabolism-related gene CPT-1 was significantly higher in the breast ($P = 0.0205$) and leg muscle ($P = 0.0468$) of high body weight chickens (Fig. 5C). IHC results showed that P-AMPK was mainly distributed in the connective tissue of breast and leg muscle, and the protein expression of P-AMPK was significantly higher in breast muscle ($P = 0.0225$) (Fig. 5D) and leg muscle ($P = 0.0153$) (Fig. 5E) of high body weight chickens. Western blot results also revealed higher protein expression of P-AMPK in breast muscle ($P = 0.0001$) (Fig. 5F and G) and leg muscle ($P = 0.0016$) (Fig. 5H and I) of high body weight chickens.

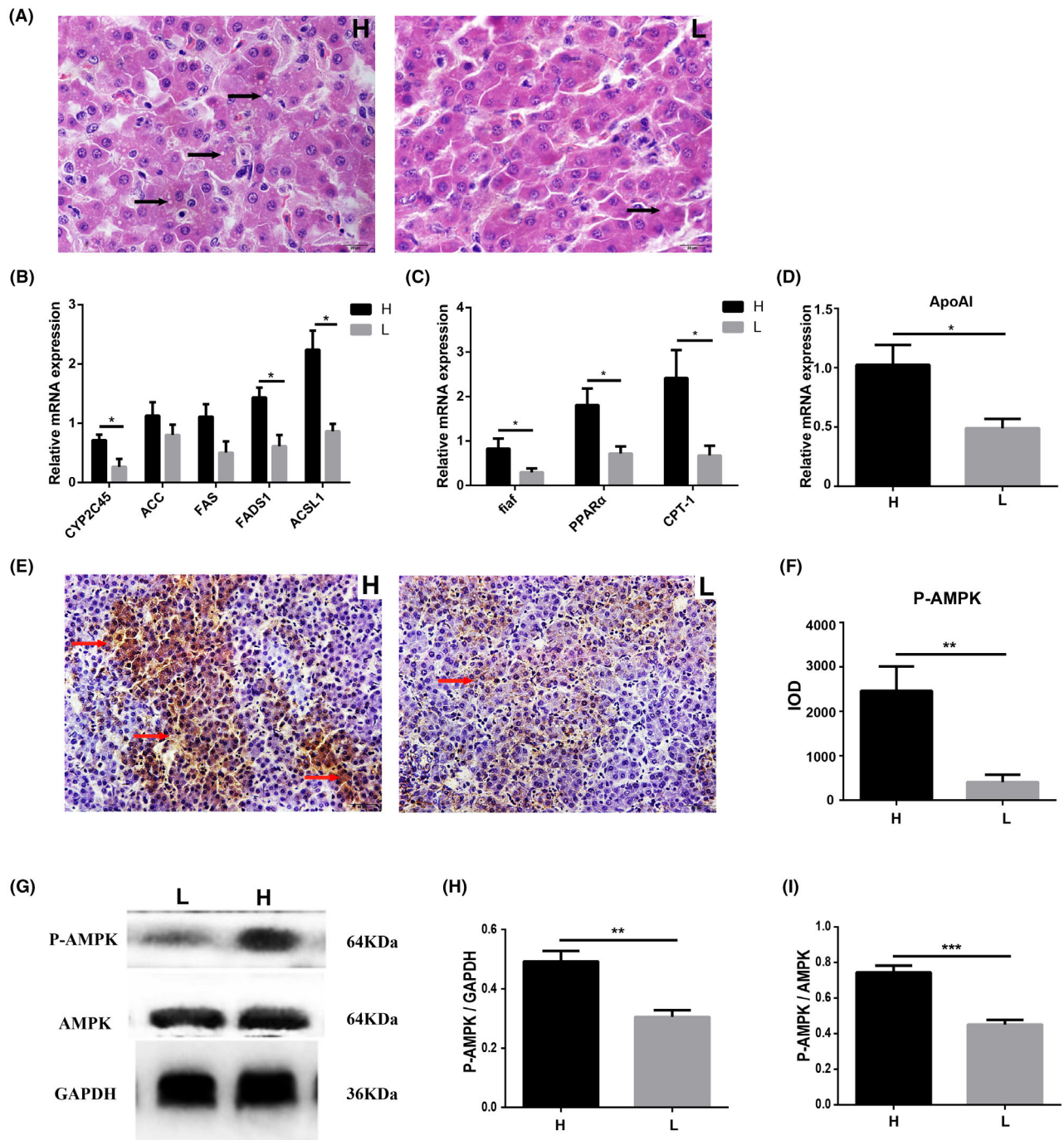


Fig. 3. Differences of fat metabolism levels in liver between high and low body weight chickens. Chickens (Turpan cockfighting × White Leghorn) at 7th week of age were sacrificed to analyse fat metabolism level in liver ($n = 10$). Each group contained five males and five female chickens.

A. Fat content of hepatocytes (H & E staining). The white dots indicated by the arrows in the figure are fat droplets.

B. The relative mRNA expression of fat anabolism-related genes (qPCR).

C. The relative mRNA expression of fat catabolism-related genes (qPCR).

D. The relative mRNA expression of fat transport-related genes (qPCR).

E. The distribution of P-AMPK (IHC). The arrows in the figure indicated positive expression of P-AMPK.

F. IOD comparison of IHC.

G–I. The protein expression of P-AMPK (western blot). Scale bars = 50 μ m. All data were presented as the means \pm SEM. P values were calculated using Student's t -test, * $P < 0.05$, ** $P < 0.01$.

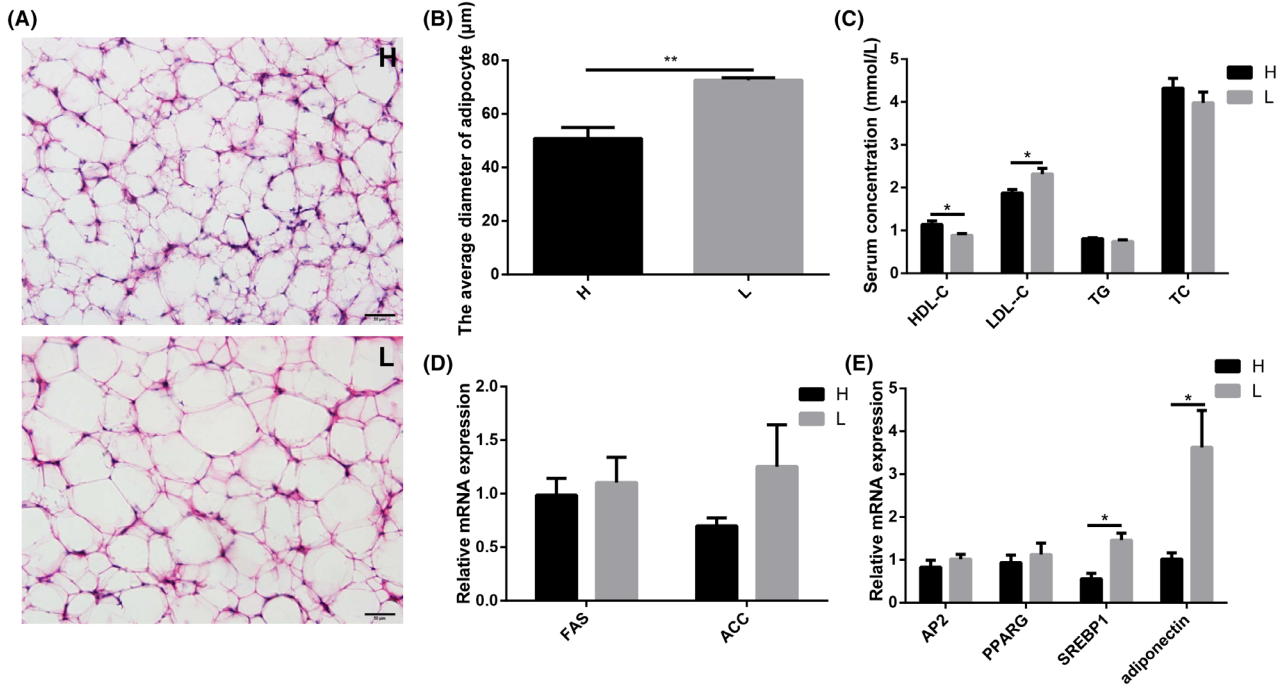


Fig. 4. Different fat metabolism levels in blood and abdominal adipose tissue.

A. Chickens (Turpan cockfighting × White Leghorn) at 7th week of age were sacrificed to analyse fat metabolism level in blood and abdominal adipose tissue ($n = 10$). Each group contained five male and five female chickens. Different adipocyte structure by H & E staining.

B. The average diameter of adipocyte.

C. Different blood biochemical parameters related to lipid metabolism.

D. The relative mRNA expression of fat anabolism-related genes in abdominal adipose tissue (qPCR).

E. The relative mRNA expression of adipocyte differentiation-related genes in abdominal adipose tissue (qPCR). Scale bars = 50 μm. All data were presented as the means ± SEM. P values were calculated using Student's t -test, * $P < 0.05$, ** $P < 0.01$.

Differential gut microbiota was related to fat metabolism in chickens

Spearman's correlation analysis was used to analyse the correlation between fat metabolism and the differential gut microbiota. The results indicated that the abundance of *Sphingomonas* and *Microbacterium* was significantly and positively correlated with fat catabolism in serum, liver, breast muscle and leg muscle. The abundance of *Slackia* was significantly and negatively correlated with the fat catabolism in serum, liver, breast muscle and leg muscle (Fig. 6).

Faecal microbiota transplantation improved growth performance and hepatic fat metabolism in chicken

Faecal microbiota transplantation (FMT) was performed to investigate the effect of gut microbiota on growth performance and fat metabolism. The results showed that the body weight in FMT group was significantly ($P < 0.05$) higher than that in control group (Fig. 7A). The relative mRNA expression of fat synthesis-related gene (Fig. 7B), fat catabolism-related gene (Fig. 7C) and fat transport-related gene (Fig. 7D) in liver was significantly ($P < 0.05$) up-regulated in FMT group than the

Fig. 5. Different fat metabolism levels in muscle tissues. Chickens (Turpan cockfighting × White Leghorn) at 7th week of age were sacrificed to analyse fat metabolism level in muscles ($n = 10$). Each group contained five male and five female chickens.

A. The relative mRNA expression of fat anabolism-related genes (qPCR).

B. The relative mRNA expression of fat transport-related genes (qPCR).

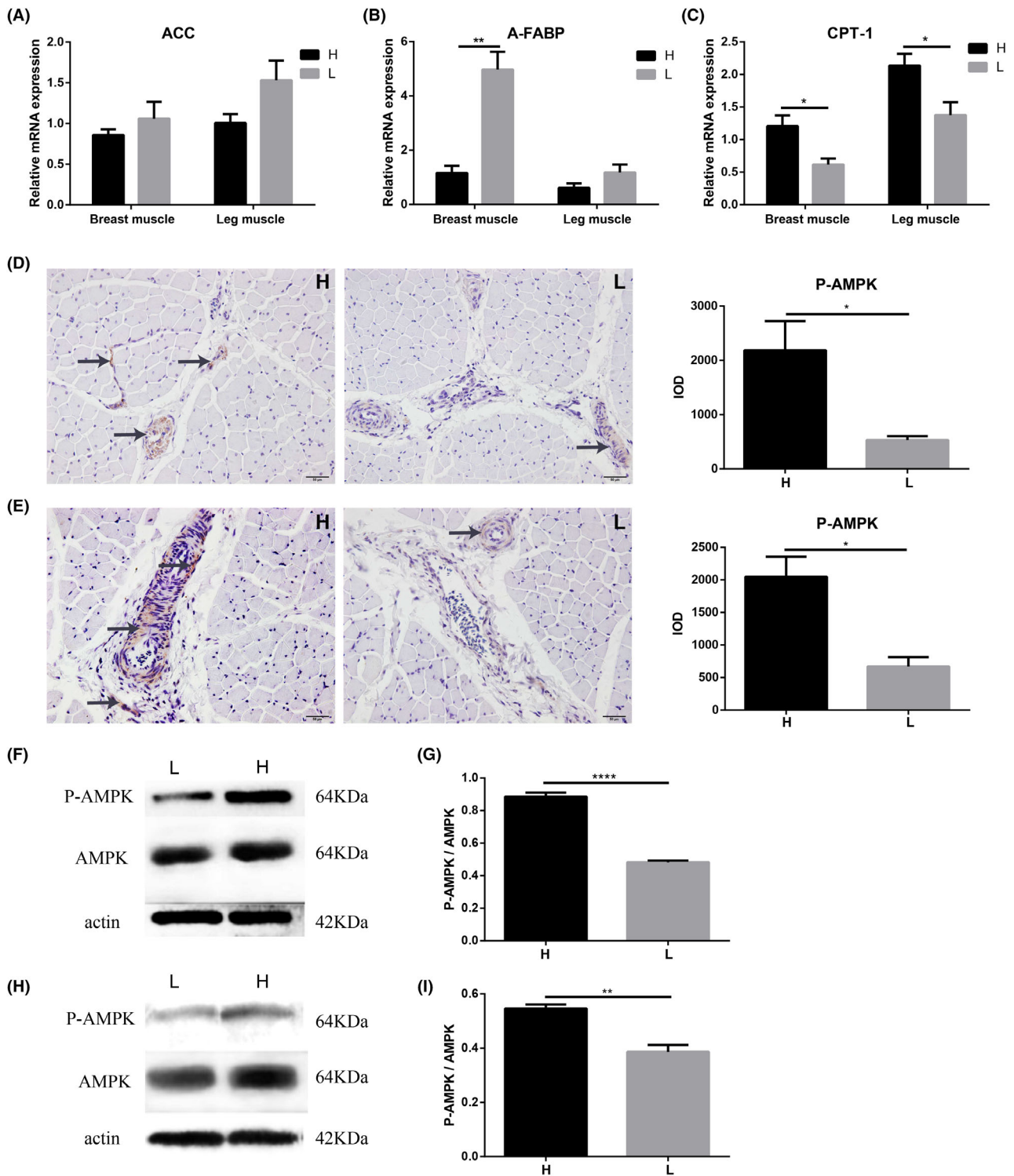
C. The relative mRNA expression of fat catabolism-related genes (qPCR). (D) The distribution and protein expression of P-AMPK in breast muscle (IHC).

E. The distribution and protein expression of P-AMPK in leg muscle (IHC). The arrows in the figures indicated positive signals.

F and G. The protein expression of P-AMPK in breast muscle (western blot).

H and I. The protein expression of P-AMPK in leg muscle (western blot). H represented high body weight chickens and L represented low body weight chickens. Scale bars = 50 μm. All data were presented as the means ± SEM. P values were calculated using Student's t -test,

* $P < 0.05$, ** $P < 0.01$.



control group. 16S rRNA gene sequencing of FMT and control groups were also performed. For FMT and control groups, we obtained 995 072 high-quality reads from 20 samples (an average of 49 753 reads per sample)

that were clustered into 920 operational taxonomic units (OTUs) at 97% sequence similarity. These results revealed that alpha and beta diversities were significantly ($P < 0.05$) different in both groups (Fig. 8A–C). At

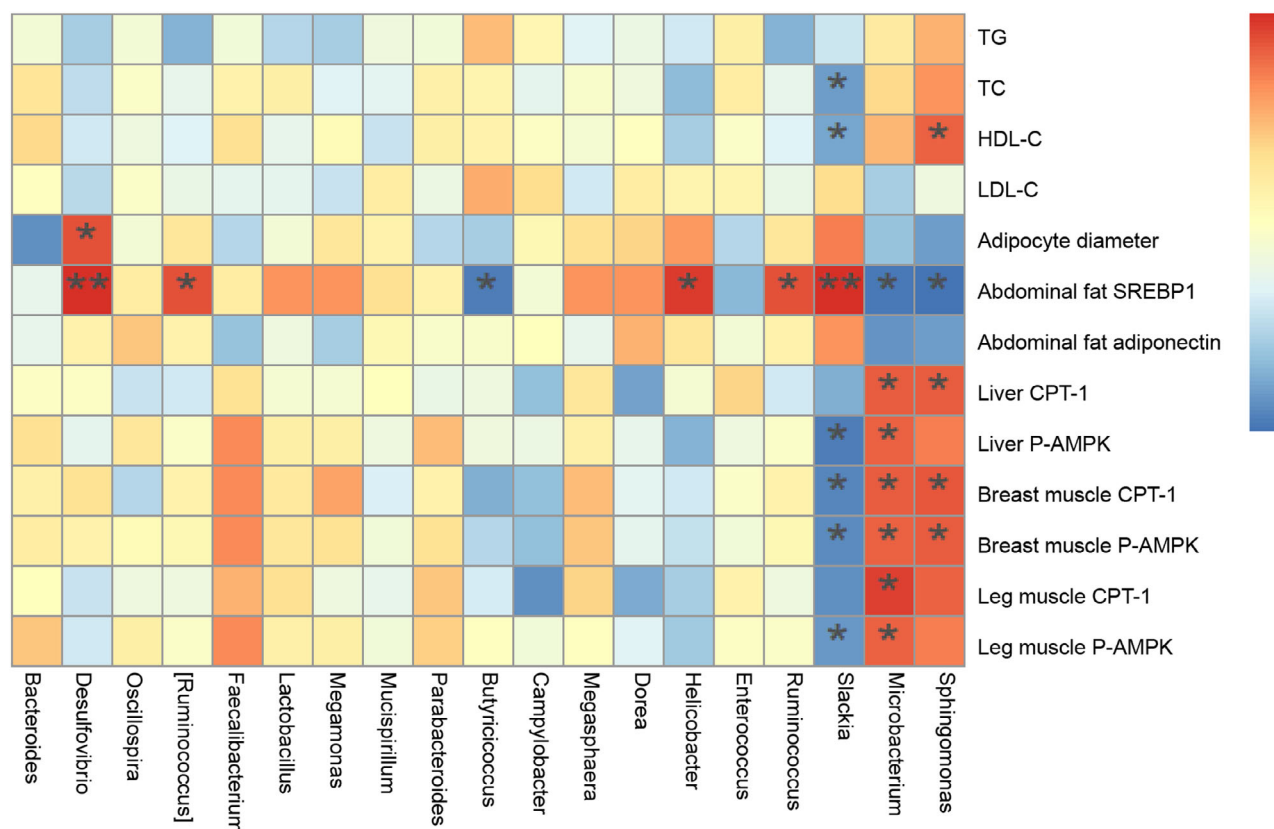


Fig. 6. Heatmap of Spearman's correlations between caecal microbe abundance and fat metabolism. The colours range from blue (negative correlation) to red (positive correlation). P values were calculated using Student's t -test, * $P < 0.05$ and ** $P < 0.01$.

the phylum level, Firmicutes, Bacteroidetes and Proteobacteria were observed as the most abundant phyla (Fig. 8D). The ratio of Firmicutes to Bacteroidetes was significantly different between FMT and control groups (Fig. 8E). LEfSe analysis revealed that the relative abundance of gut microbiota was also significantly ($P < 0.05$) different in FMT and control groups (Fig. 8G).

Discussion

The fat metabolic profile associated with gut microbiota is a strong determinant to regulate host physiology. The current study aimed to investigate the critical role of gut microbiota in the growth performance of chicken via regulating fat metabolism.

Fat metabolism has considerable significance in biological processes. It is an essential and complex biochemical reaction, including digestion, absorption, synthesis and catabolism. Digested fat in the form of glycerol and fatty acid is absorbed into the bloodstream and transported to the liver, adipose tissue and other organs (Hermier, 1997; Margetak *et al.*, 2012). Fat synthesized in the liver is bound to apolipoprotein or cholesterol to form very low-density lipoprotein (VLDL), which

is transported through blood to other tissues for storage or usage (Alves-Bezerra and Cohen, 2017). However, fat synthesized in adipose tissue is stored there. In chickens, adipose tissue plays a vital role to regulate homeostasis and metabolic energy (Di Somma *et al.*, 2019). Blood biochemical indicators are also closely related to fat metabolism and have been used as criteria to select lean chicken lines (Zhang *et al.*, 2018). In the present study, significantly higher concentrations of HDL-C were observed in high body weight chickens, suggesting that it might effectively carry cholesterol through blood and transport it to the liver (Oldoni *et al.*, 2014; Manthei *et al.*, 2018).

The liver is the largest solid organ and plays a critical role in lipid metabolism, providing significant energy resources for host growth (Grijalva and Vakili, 2013; Parry and Hodson, 2017). The chicken liver is reported as the leading site for de novo synthesis of fatty acid (Mellouk *et al.*, 2018). Hepatic lipid metabolism effectively regulates the fat synthesis and catabolic related genes (Na *et al.*, 2018). In our study, high body weight chickens exhibited higher expression of fat synthesis-related genes (P450 2C45 (CYP2C45), FADS1 and ACSL1) in the liver, as well as greater numbers of fat vacuoles in hepatocytes,

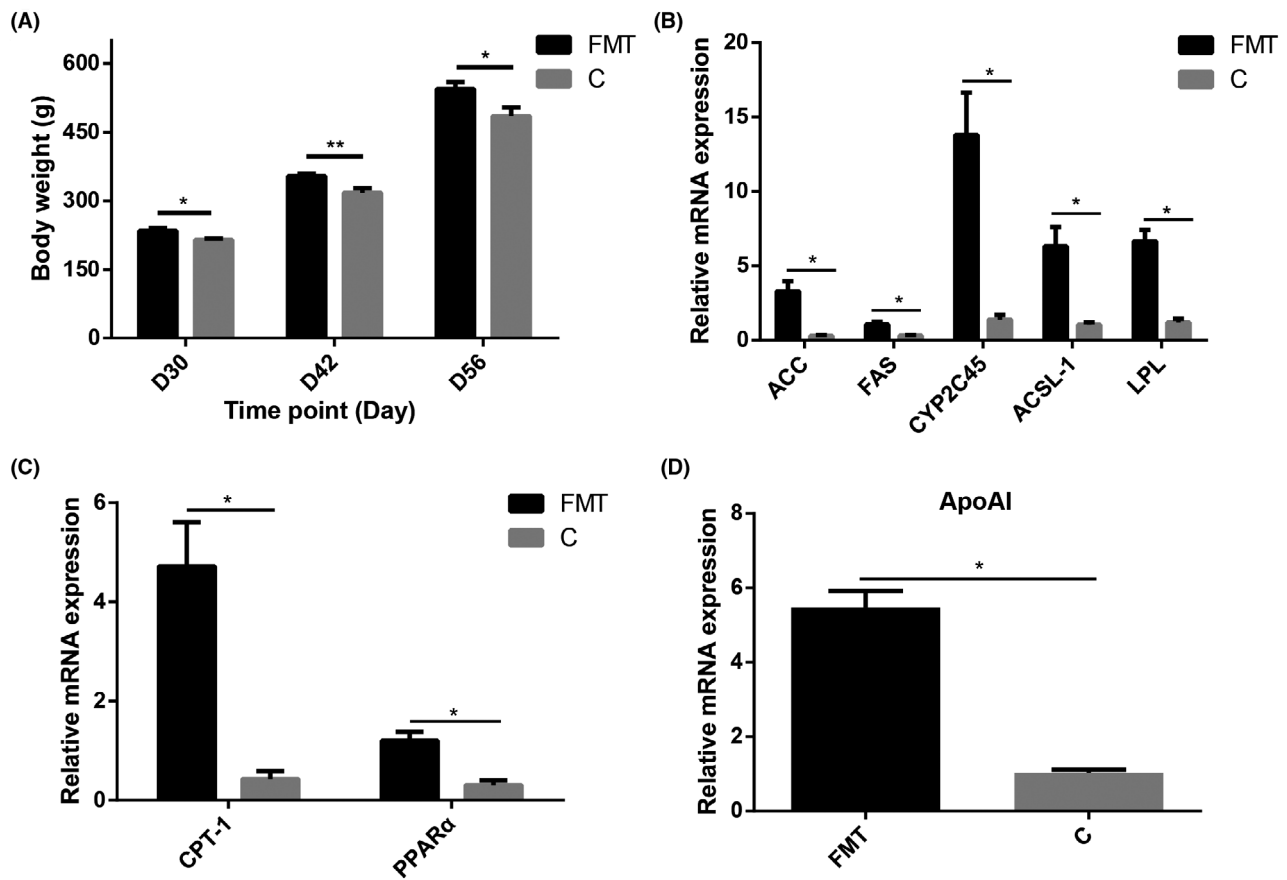


Fig. 7. FMT influenced growth performance and fat metabolism level in liver.

Newly hatched chicks (Turpan cockfighting \times White Leghorn) of FMT group and control group (C) were orally administrated with 1 ml faecal microbiota suspension and saline, respectively, for 30 days and sacrificed at the age of 31 days to analyse fat metabolism level in liver ($n = 10$).

A. Comparison of body weight between C and FMT group.

B. The relative mRNA expression of fat anabolism-related genes (qPCR).

C. The relative mRNA expression of fat catabolism-related genes (qPCR).

D. The relative mRNA expression of fat transport-related genes (qPCR). All data were presented as the means \pm SEM. P values were calculated using Student's t -test, * $P < 0.05$, ** $P < 0.01$.

suggesting a potent fat synthesis in the livers of high body weight chickens. At the same time, the expression of fat catabolism-related genes (f1af, PPAR α and CPT-1) increased, ultimately promoting the oxidative catabolism of fatty acids, thereby providing energy for the growth and development of chickens (Witczak *et al.*, 2008; Niu *et al.*, 2010; Kim *et al.*, 2017). Significantly higher expression of fat transport-related hepatic ApoA1 gene in high body weight chickens also indicated an appropriate regulation in fat transport (Liu *et al.*, 2016; Zheng *et al.*, 2016). Additionally, a remarkably prominent expression of hepatic phosphorylated-AMPK in hepatocytes of higher body weight chickens maintained the energy balance through fatty acid synthesis and oxidation (Yang *et al.*, 2018; Hu *et al.*, 2019a; Li *et al.*, 2020a). Abdominal adipose tissue is the main organ of fat synthesis and deposition. The up-regulated expression of adipocyte differentiation-related

genes can mediate excessive proliferation and differentiation of adipocytes, causing excessive deposition of fat in animal bodies (Xiao *et al.*, 2018). Abdominal fat deposition impacts negatively on carcass quality and feed efficiency (Demeure *et al.*, 2013). In the present study, the expression of fat synthesis- and adipocyte differentiation-related genes in low body weight chickens was significantly higher than in high body weight chickens, suggesting increased fat synthesis in abdominal adipose tissue. In addition, the average diameter of adipocytes in low body weight chickens was much larger, indicating that more synthesized fat was stored in abdominal adipose tissue in low body weight chickens. As a result, excessive fat deposition affected growth performance (Xiao *et al.*, 2018). These results indicated that the differential fat metabolism level is a key factor leading to differences in growth performance.

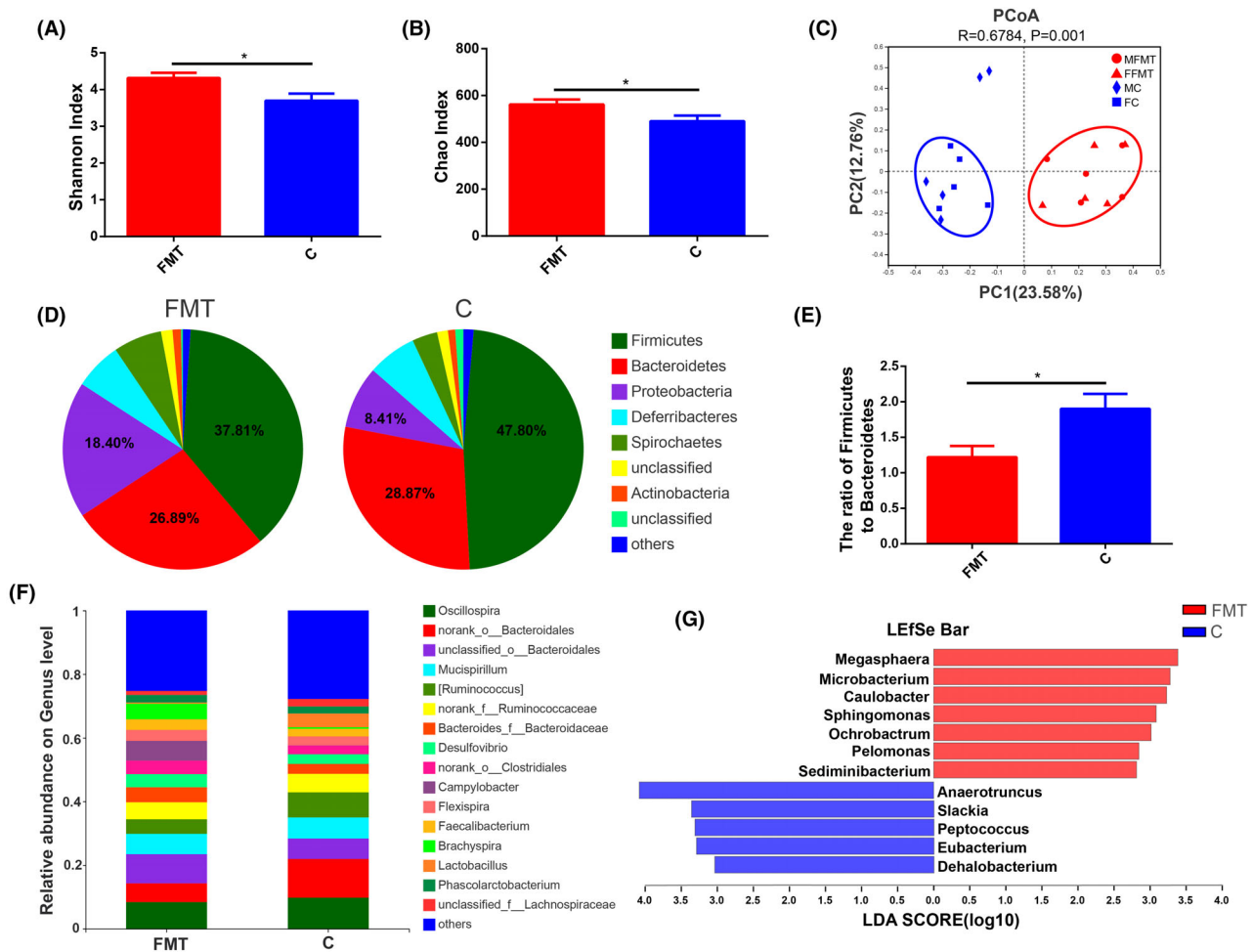


Fig. 8. Differences in caecal microbiota between FMT and control groups.

A. Chickens (Turpan cockfighting × White Leghorn) at 31st day of age were sacrificed to perform 16S rRNA sequence of caecal microbiota ($n = 10$). Each group contained five males and five female chickens. Microbial community diversity (Shannon index).

B. Microbial community abundance (Chao index).

C. Beta diversity of FMT and control groups. MFMT: male in FMT group, FFMT: female in FMT group, MC: male in control group, FC: female in control group.

D. The composition of microbial communities at the phylum level.

E. The ratio of Firmicutes to Bacteroidetes.

F. The composition of microbial communities at the genus level.

G. LDA score for LefSe analysis (LefSe was done at LDA score of >2).

It has been reported that the composition of the gut microbiota is closely related to fat metabolism in mammals and chickens (Ridaura *et al.*, 2013; Yang *et al.*, 2016; Pascale *et al.*, 2019; Wen *et al.*, 2019). In human and mice, obese individuals had a lower relative abundance of the Bacteroidetes and higher relative abundance of the Firmicutes than lean controls (Ley *et al.*, 2006; Turnbaugh *et al.*, 2008). The ratio of Firmicutes/Bacteroidetes is vital for optimum nutritional physiology (De Filippo *et al.*, 2010; Bervoets *et al.*, 2013). Further findings indicated that the higher ratio of Firmicutes and Bacteroidetes can induce fat deposition (Ley *et al.*, 2006; Compare *et al.*, 2016; Koliada *et al.*, 2017; Indiani *et al.*,

2018). In addition, recent studies described *Sphingomonas* as an abundant bacterium in the gut microbiota of chicken (Chen *et al.*, 2018a; Li *et al.*, 2020b). Another study reported that sphingolipids from *Sphingomonas* could control different cellular processes, that is migration, apoptosis and proliferation (Bryan *et al.*, 2016). For instance, *Sphingomonas* synthesized sphingosine, which later with fatty acids derivatives formed sphingomyelin, thus reduced fat deposition in the chicken liver. These findings suggested that the abundance of *Sphingomonas* genus was significantly correlated with hepatic fat metabolism (Li *et al.*, 2020b). *Microbacterium* and *Sphingomonas* related to lipid metabolism were significantly

observed across all chicken embryonic stages, which suggested a significant contribution in the development of gut microbiota for chicken growth (Akinyemi *et al.*, 2020). In another study, the clusters of orthologous groups (COG) analysis revealed 20 lipid metabolism genes associated with *Microbacterium* (Chen *et al.*, 2018b). A few other studies also reported that *Microbacterium* was important in lipid metabolism (Hadjadj *et al.*, 2016; Akinyemi *et al.*, 2020). *Slackia* could also be involved in an unbalanced compositional signature of gut microbiota. Therefore, its role in imbalancing gut microbiota and disease susceptibility is critical. Contradictory findings have been reported in different studies about *Slackia*. For instance, a recent study reported that *Slackia* isolated from chicken intestine was the best detoxifying agent against mycotoxin and helps improve chicken intestinal functions (Gao *et al.*, 2020). However, several other studies reported that *Slackia* was found to be significantly enriched in the microbiota of gastric carcinogenesis and not only caused disease progression but also gut dysbiosis (Coker *et al.*, 2018; Kharrat *et al.*, 2019). Another recent study also reported that *Slackia*-related lipid metabolism abnormality may abruptly change the levels of HDL, LDL and Apo A (Han *et al.*, 2019). In the current study, higher Firmicutes/Bacteroidetes ratio in low body weight group revealed that chickens in this group stored more fat in reservoir instead of using it as energy for their growth. *Sphingomonas*, *Microbacterium* and *Slackia* were significantly different in high and low body weight chickens. Spearman's analysis demonstrated positive correlations between HDL-C, CPT-1, and P-AMPK and the relative abundances of *Sphingomonas* and *Microbacterium* and negative correlations between HDL-C, CPT-1, and P-AMPK and the relative abundance of *Slackia*. These results indicated that *Sphingomonas* and *Microbacterium* might promote the oxidative decomposition of fatty acids to stimulate growth and development of animals, yet *Slackia* promoted the deposition of fat in animals.

FMT is an effective way to reshape gut microbiota (Metzler-Zebeli *et al.*, 2019; Guo *et al.*, 2020). Transferring faecal microbiota from adult chickens with high body weight into one-day-old chicks was performed to verify whether chicken gut microbiota affected growth performance by regulating fat metabolism. The body weight in FMT group was significantly higher than that in control group. The relative mRNA expressions of hepatic anabolic (ACC, FAS, CYP2C45, ACSL1 and LPL), catabolic (PPAR α and CPT-1) and transportation (ApoA1) fat genes were significantly up-regulated. The ratio of Firmicutes to Bacteroidetes was significantly different between FMT and control groups. The relative abundance of *Sphingomonas* and *Microbacterium* was significantly higher in FMT group, yet *Slackia* was

significantly higher in control group. The results indicated that FMT increased chicken growth performance by remodelling gut microbiota which could regulate fat metabolism.

Conclusion

In summary, the levels of fat metabolism in liver, abdominal adipose tissue, breast muscle and leg muscle differ between high and low body weight chickens. *Sphingomonas*, *Microbacterium* and *Slackia* were significantly different in high and low body weight chickens. FMT improved chicken growth performance and changed fat metabolism level in liver. These findings provided novel insights into the gut microbiota increased chicken growth performance by regulating fat metabolism and contributed to the development of alternatives to AGPs for improving chicken productive efficiency.

Experimental procedures

Animals

The Institutional Animal Care and Use Committee of Huazhong Agricultural University approved all the animal procedures, and all methods were performed in accordance with the relevant guidelines and regulations.

Newly hatched chickens (Turpan cockfighting \times White Leghorn chickens for both meat and egg) were reared under the same husbandry conditions and were fed a corn-soybean diet in pellet form in a poultry farm with no medication or vaccination. The birds had *ad libitum* access of water and feed. When they were 7 weeks old, a total of 20 chickens with the highest ($n = 10$, five males and five females) and the lowest ($n = 10$, five males and five females) body weights were selected for the next study. In faecal microbiota transplantation experiment, two adult female chickens (ranked 5th and 6th), which harboured the top 10 body weight in the same batch, with no gastrointestinal diseases or drug treatment, were selected as faecal donors. Fresh faecal samples were collected every day for 30 days during morning time in sterile 50-mL tube and 0.75% saline were mixed in 1:6 ratios (6 ml of 0.75% saline for each gram of faeces). Then collect the supernatant and filter it with sterile gauze to get faecal suspension. Then, a total of 60 one-day-old chickens with the same genetic background were selected as recipients. The recipients were randomly divided into two groups: saline control group (C group) and faecal microbiota transplantation group (FMT group). Each bird in FMT group was orally administrated with 1 ml faecal microbiota suspension for 30 days while the birds in control group were orally administrated with 1 ml 0.75% saline.

Samples collection

Samples were collected from the high and low body weight chickens (five males and five females in each group) at 7 weeks of age, and samples from the chickens in the FMT and control groups were collected at the age of 31st day. After fasting for 12 h, the chickens were sacrificed and blood, liver and abdominal adipose were harvested. Breast muscle and leg muscle were also collected and weighed. To ensure the comparability of research results, the same part of each organ was chosen for further analysis. For gut microbiota analysis, the contents of the caeca from the selected twenty chickens were snap-frozen in liquid nitrogen and stored at -80°C . For histo-morphological analysis, freshly harvested breast muscle, leg muscle and liver tissues were fixed in 4% paraformaldehyde solution. Similarly, abdominal adipose tissues from each chicken were fixed in optimal cutting temperature (OCT) compound. For protein and gene expression analysis, the parts of freshly harvested muscle, adipose and liver tissues were snap-frozen in liquid nitrogen and then stored at -80°C . For blood biochemical parameters analysis, blood samples from birds were centrifuged at 1500 *g* for 15 min and serum samples were snap-frozen in liquid nitrogen and stored at -80°C for subsequent analysis.

Muscle index calculation

The muscle index was calculated using the following formula: muscle index = muscle weight (g)/ body weight (g).

Microbial genomic DNA extraction and 16S rRNA gene sequencing

16S rRNA gene sequencing was used to compare the microbial composition between high and low body weight chickens. Total bacterial genomic DNA was extracted from samples using Fast DNA SPIN extraction kits (MP Biomedicals, Santa Ana, CA, USA), following the manufacturer's instructions and as described by (Hu *et al.*, 2019b). The sample (500 mg) was mixed in Lysing Matrix E tube with 978 μl sodium phosphate buffer and MT buffer (122 μl). Then, the mixture was homogenized at 6.0 m s^{-1} speed for 40 s and centrifuged for 10 min at 14 000 *g*. The obtained supernatant was mixed with protein precipitation solution (250 μl) in a clean 2.0-ml micro-centrifuge tube. This mixture was mixed thoroughly in the tube, 10 times by shaking with hands. To remove the protein, the mixture was again centrifuged for 5 min at 14 000 *g*. Subsequently, the supernatant was transferred to 15-ml tube, 1 ml Binding Matrix Solution was added, suspended it for 2 min and placed the tube on a rack for 3 min. After discarding 500 μl of supernatant,

600 μl of DNA Solution was transferred to a SPINTM Filter Tube followed by centrifuging for 1 min at 14 000 *g*. Then, 500 μl SEWS-M wash Solution was added and centrifuged for 1 min at 14 000 *g*. To remove the residual SEWS-M wash solution, it was again centrifuged for 2 min at 14 000 *g* followed by drying for 5 min at room temperature. Finally, 50 μl of DES was added for the resuspension of binding matrix and centrifuged for 1 min at 14 000 *g* to obtain DNA. The extracted DNA was stored at -20°C for further analysis. The quantity and quality of extracted DNA fragments were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis respectively.

PCR amplification of the bacterial 16S rRNA gene V3-V4 region was performed using the forward primer 338F (5'-ACTCCTACGGGAGGCAGCA-3') and the reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Sample-specific 7-bp barcodes were incorporated into the primers for multiplex sequencing. The PCR components contained 5 μl of Q5 reaction buffer (5 \times), 5 μl of Q5 High-Fidelity GC buffer (5 \times), 0.25 μl of Q5 High-Fidelity DNA Polymerase (5 U μl^{-1}), 2 μl (2.5 mM) of dNTPs, 1 μl (10 μM) of each forward and reverse primer, 2 μl of DNA Template and 8.75 μl of dd H₂O. Thermal cycling consisted of an initial denaturation at 98 $^{\circ}\text{C}$ for 2 min, followed by 25 cycles of denaturation at 98 $^{\circ}\text{C}$ for 15 s, annealing at 55 $^{\circ}\text{C}$ for 30 s and extension at 72 $^{\circ}\text{C}$ for 30 s, with a final extension of 5 min at 72 $^{\circ}\text{C}$. PCR amplicons were purified with Agencourt AMPure Beads (Beckman Coulter, Indianapolis, IN) and quantified using the PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). After the individual quantification step, amplicons were pooled in equal amounts, and paired-end 2 \times 300 bp sequencing was performed using the Illumina MiSeq platform with the MiSeq Reagent Kit v3 at Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China).

Sequencing data analysis

The Quantitative Insights Into Microbial Ecology (QIIME, v1.8.0) pipeline was employed to process the sequencing data, as previously described by (Caporaso *et al.*, 2010). Briefly, raw sequencing reads with exact matches to the barcodes were assigned to respective samples and identified as valid sequences. The low-quality sequences were filtered through the following criteria (Gill *et al.*, 2006; Chen and Jiang, 2014): sequences that had a length of < 150 bp, sequences that had average Phred scores of < 20, sequences that contained ambiguous bases and sequences that contained mononucleotide repeats of > 8 bp. Paired-end reads were assembled using Fast Length Adjustment of SHort reads

(FLASH), a fast computational tool (Magoč and Salzberg, 2011). After chimera detection, the remaining high-quality sequences were clustered into Operational Taxonomic Units (OTUs) at 97% sequence identity by UCLUST (Edgar, 2010). A representative sequence was selected from each OTU using default parameters. OTU taxonomic classification was performed with BLAST, searching the representative sequences set against the Greengenes Database (DeSantis *et al.*, 2006) using the best hit method (Altschul *et al.*, 1997). Further, to record the abundance and taxonomy of these OTUs in every sample, an OTU table was generated. OTUs containing less than 0.001% of total sequences across all samples were discarded. To minimize the difference in sequencing depth across samples, an average, rounded rarefied OTU table was made. As this table was based on taking an average of 100 evenly resampled OTU subsets under 90% of the minimum sequencing depth, so, it was used for further analysis.

Blood biochemical parameters analysis

To test the fat metabolism level in blood, the concentrations of serum TG, TC, HDL-C and LDL-C were determined using a Rayto Chemistry Analyzer Chemray 240 (Chemray 240, China) according to the manufacturer's instructions with the commercial diagnostic kits (Changchun Huili Biotec. Co., Ltd). The serum sample was mixed thoroughly with reaction solution in recommend ratio and kept at 37 °C for 10 min. After that the absorbance was measured and total concentration was calculated following the formula: Total concentration = Absorbance of sample / Absorbance of calibration solution × Calibration concentration (mmol l⁻¹).

Haematoxylin and eosin (H & E) staining

To compare the morphological changes, liver, breast muscle and leg muscle tissue samples, embedded in paraffin, were cut into 3-μm-thick sections with a rotary slicer (LEICARM2245, Leica, Germany). Abdominal adipose tissue samples fixed in OCT were cut into 10-μm-thick frozen slice with a microtome cryostat (Shandon Cryotome FSE, Thermo Fisher Scientific, Waltham, MA, USA). H & E staining was performed using a routine protocol, and the examination of stained tissue sections was accomplished by light microscopy (Olympus BX51, Tokyo, Japan) with a digital camera (DP72; Olympus). The average diameter of adipocytes and the average areas of breast muscle cells and leg muscle cells were quantitated using Image-Pro Plus 6.0.

Immunohistochemical (IHC) staining

To test the distribution and protein expression of P-AMPK, immunohistochemical staining was performed following the same steps as described in earlier studies (Ansari *et al.*, 2016; Rahman Ansari *et al.*, 2016). Briefly, the tissue sections were deparaffinized twice in xylene and rehydrated in a graded series of ethanol. A microwave oven (MYA-2270M, Haier, Qingdao, China) was used for heat antigen retrieval in citrate acid buffer solution (pH 6.0) for 20 min (5 min at high level, i.e. 700 W and 15 min at low level, i.e. 116 W). After cooling at room temperature for 2–3 h, 3% H₂O₂ was used to block endogenous peroxidase. For blocking of non-specific antibody binding, the tissue sections were incubated with 5% bovine serum albumin (BSA) at 37 °C for 30 min. Sections were then incubated with primary antibodies using rabbit anti-P-AMPK antibody (1:100) (Cell Signaling Technology, Inc., USA). Subsequently, tissue sections were incubated at 37 °C with suitable horseradish peroxidase (HRP)-conjugated secondary antibodies (Boster, Wuhan, China) for 30 min. Immunostaining for all the tissue sections was accomplished using the chromogenic marker Diaminobenzidine (DAB) (Boster, Wuhan, China), and counterstaining was performed using haematoxylin. Then, the sections were washed, dried, dehydrated, cleared and finally mounted with a coverslip.

Serial sections were examined under a light microscope (BH-2; Olympus, Japan) with a digital camera (DP72; Olympus), and the fields of vision were chosen according to different regions of the liver and muscle tissue in each section. The distributions and expression levels of different proteins were measured in high-power fields selected at random. All of the images were taken using the same microscope and camera set. Image-Pro Plus (IPP) 6.0 software (Media Cybernetics, USA) was used to calculate the mean density for positive staining.

Western blotting (WB)

To test the protein expression of P-AMPK, Western blotting was performed following previously described methods (Hnasko and Hnasko, 2015). Briefly, the frozen specimens were powdered in liquid nitrogen and homogenized in lysis buffer with a protease inhibitor. The supernatants were vortexed, incubated on ice and centrifuged at 12 000 *g* for 5 min. Protein concentrations were measured using the BCA protein quantification kit (Beyotime, Jiangsu, China). Equal amounts of total proteins (40 μg) were subjected to 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (30 min at 80 volts, following 80 min at 120 volts). Then, the separated proteins were transferred onto a polyvinylidene difluoride (PVDF)

membrane (Merck Millipore, USA). The membranes were incubated with rabbit anti-P-AMPK (1:1000) (Cell Signaling Technology, Inc., USA), rabbit anti-AMPK (1:1000) (ABclonal, China), mouse anti-GAPDH (1:10 000) (Proteintech Group, Inc., USA) and rabbit anti- β -actin (1:5000) (ABclonal, China) antibodies for 12 h. After washing in 1X TBST buffer three times, samples were incubated with peroxidase-conjugated secondary antibody (1:5000) for 120 min (Boster, China). The blots were developed with Super Signal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA) and visualized using ChemiDoc-It™ Imaging System. Western blot results were analysed using IPP 6.0 software.

Real-time quantitative polymerase chain reaction (qPCR)

To measure the expression of fat metabolism-related genes at the mRNA level, total RNA was isolated from the liver, abdominal adipose tissue, breast muscle and leg muscle with TRIzol reagent (Takara, Japan) according to the manufacturer's instructions. The cDNA was synthesized using the RevertAid First-Strand cDNA Synthesis Kit (Takara, Japan). The reaction mixture (10 μ l) for qPCR contained 5 μ l of SYBR Select Master Mix for

CFX (Takara, Japan), 0.4 μ l of each forward and reverse primer, 3.2 μ l of ddH₂O and 1 μ l of template cDNA. The qPCR reactions were performed on a Bio-Rad CFX Connect real-time qPCR detection system (Bio-Rad, Hercules, CA, USA).

The qPCR conditions were as follows: pre-denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and elongation at 72 °C for 20 s. The primer sequences are listed in Table 1. β -Actin was chosen as a reference for qPCR. All samples were run in triplicate, and gene expression levels were quantified using the $\Delta\Delta$ Ct method.

Statistical analysis

Sequence data analyses were mainly performed using QIIME and R packages (v3.2.0). OTU-level alpha diversity indices, such as the Chao richness index and Shannon diversity index, were calculated using the OTU table in QIIME. The taxonomic compositions and abundances were visualized using Excel. LEfSe was performed to detect differentially abundant taxa across groups using the default parameters (LDA > 2) (Segata *et al.*, 2011). Spearman's correlations between the gut microbiota and

Table 1. Primers used for real-time qPCR

| Gene | Primer sequences (5' to 3') | Accession no. |
|----------------|--|----------------|
| β -actin | f-TTGTTGACAATGGCTCCGGT r-TCTGGGCTTCATCACCAACG | NM_205518.1 |
| ApoA1 | f-GTGACCCTCGCTGTGCTCTT r-CACTCAGCGTGCCAGGTTGT | NM_205525.4 |
| LPL | f-TGGACATTGGTGACCTGCTTATGC r-TCGCCTGACTTCACTCTGACTCTC | NM_205282.1 |
| ACC | f-TCCAGCAGAACCGCATTGACAC r-GTATGAGCAGGCAGGACTTGGC | NM_205505.1 |
| FAS | f-GCTCTGCGTCTGCTTCACTCTAC r-GGTACAGGACTCTGCCATCAATGC | NM_001199487.1 |
| FADS1 | f-CCGTGCCACTGTGGAGAAGATG r-GCCTAGAAGCAACGCAGAGAAGAG | LC061145.1 |
| CYP2C45 | f-AACAAGCACCACCACACGATACG r-GGTCAGCCACGCAAGGTCTTC | AJ430583.1 |
| ACSL1 | f-GACTAATGGTACACAGGAGCAGCAC r-CCAGGCATTGACAGTGAGCATCC | NM_001012578.1 |
| PPAR α | f-TGCTGTGGAGATCGTCCTGGTC r-CTGTGACAAGTTGCCGGAGGTC | AF163809.1 |
| CPT-1 | f-GCCAAGTCGCTCGCTGATGAC r-ACGCCTCGTAGGTCAGACAGAAC | DQ314726.1 |
| fiaf | f-AGATCAAGCAGCAGCAGTACAAGC r-ACGCTCACATTATGGCTCTGGTTG | XM_001232283.5 |
| A-FABP | f-ACAATGGCACACTGAAGCAGG r-AGCAGGTTCCCATCCACCAC | FJ493543.1 |
| SREBP1 | f-GGTCCGGGCCATGTTGA r-CAGGTTGGTGCGGGTGA | AJ310768.1 |
| PPARG | f-GAATGCCACAAGCGGAGAAGGAG r-GCTCGCAGATCAGCAGATTCAGG | NM_001001460.1 |
| AP2 | f-ACTGAAGCAGGTGCAGAAGTGG r-TGCATTCCACCAGCAGGTTCC | NM_204290.1 |
| Adiponectin | f-TACGTGTACCGCTCCGCCTTC r-GTGCTGCTGCTAGTGGTTCTG | KP729052.1 |

fat metabolism were determined using the R software package. All data are presented as the means \pm standard error of mean (SEM). All analyses and graphic representations were performed with Prism software 5.01 (GraphPad Software, Inc., San Diego, CA, USA). The statistical significance of the mean values in two-group comparisons was determined using Student's *t*-test. A *P* value < 0.05 was considered statistically significant.

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Conflict of interest

The authors declare that they have no conflicts of interest.

Author contributions

XL Zhang, YF Hu and HZ Liu designed the research. XL Zhang, YF Hu, Y Chen, RR Cheng, L Cui and AA Nafady performed the research. XL Zhang, YF Hu, AA Elokil and HZ Liu analysed the data. XL Zhang, YF Hu, AR Ansari, M Akhtar, El-SM Abdel-Kafy and HZ Liu wrote the paper with the help of all authors. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The current scientific investigation was conducted in accordance with the rules and regulations of the ethics committee for use of animals, Huazhong Agricultural University (HZAUCH-2018-008), Wuhan, China.

Consent for publication

Not applicable.

Data availability statement

The raw 16S rRNA gene sequencing data are available at the NCBI Sequence Read Archive (SRA), under BioProject PRJNA637407.

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