

# Effects of Eubiotic Lignocellulose on the Gut Microbiota and Metabolism of Chickens

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## Research

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# Abstract

**Background:** Dietary fiber is an important factor shaping the gut microbiota. Eubiotic lignocellulose is a useful source of dietary fiber for chickens; however, research on its effects on the gut microbiota and metabolism of chickens is limited. Moreover, the gut microbiota regulates the host metabolism, which generally depend on microbial metabolites—short-chain fatty acids (SCFAs) and the SCFA receptors; however, related research reports on the SCFA receptors of chickens are lacking. Given this, we added three different levels of eubiotic lignocellulose (0%, 1%, and 2%) to the feed of ISA brown hens (IBH) for 0–8 weeks, with the aim of observing the effects of added eubiotic lignocellulose on the gut microbiota, SCFAs and its receptors and metabolism of chickens.

**Results:** The results showed that the addition of eubiotic lignocellulose showed no significant difference among groups in terms of the growth performance, development of the cecum and microbial diversity of IBH ( $P > 0.05$ ), but significantly ( $P < 0.05$ ) increased the relative abundance of the excellent fiber-degradation bacterium *Bacteroides thetaiotaomicron*, the acetate-producing bacterium *Sutterella* and fiber digestibility. The addition of 1% eubiotic lignocellulose significantly ( $P < 0.05$ ) increased the lactate-producing bacterium *Lactobacillus panis*, the butyrate-producing bacterium *Oscillospira* and the concentration of SCFAs in the cecum chyme. The addition of eubiotic lignocellulose had no significant effect on the relative expression of the SCFA receptor–G protein-coupled receptor 43 (GPR43) mRNA in the liver and cecum ( $P > 0.05$ ), resulting in there being no significant difference ( $P > 0.05$ ) among groups in the metabolism of chickens, including gastrointestinal hormones glucagon-like peptide-1 (GLP-1), peptide tyrosine–tyrosine (PYY), blood glucose and liver glycogen.

**Conclusions:** The addition of eubiotic lignocellulose can increase the abundance of the fiber-degradation bacterium *Bacteroides thetaiotaomicron* and the fiber digestion of chickens at 8 weeks. The addition of 1% eubiotic lignocellulose is more conducive to the fiber-degradation bacteria and the SCFA-producing bacteria, resulting in increasing of the production of SCFAs. However, because the difference of the concentration of SCFAs was not great among groups, there was no significant change in the growth performance of chickens, the development of the cecum and the relative expression of GPR43 mRNA, resulting in slight effects on the metabolism, including GLP-1 and PYY and the blood glucose and liver glycogen of chickens. Increasing our understanding of this would be beneficial to providing a theoretical basis for the application of eubiotic lignocellulose and improve the growth performance and health of chickens.

## 1. Introduction

Dietary fiber is an important source for animals, however, monogastric animals such as chickens and humans must rely on gut microorganisms to degrade fiber due to a lack of endogenous fiber-degrading enzymes [1]. Dietary fiber is the substrate of microorganisms residing in the gut. Dietary fiber is broken down into monosaccharide or oligosaccharide by carbohydrate-active enzymes (CAZymes) encoded by genomes of fiber-degradation bacteria [2]. *Bacteroides* and *Prevotella* are fiber-degradation generalists in

Bacteroidetes. Bacteroidetes can break down a series of plant-derived dietary polysaccharides into monosaccharides via unique polysaccharide utilization locus (PUL) [3]. PUL encodes a hybrid two-component system (HTCS), glycoside hydrolase (GH) and other enzymes to degrade dietary fiber by cleaving glycosidic bonds [4]. In contrast, Firmicutes is regarded as the fiber-degradation specialist and it process glucose via gram-positive PULs (gpPULs) [5]. Excellent fiber-degradation genera in Firmicutes include *Ruminococcus*, *Fibrobacter*, *Butyrivibrio*, *Roseburia* and so on. *Ruminococcus* can efficiently utilize cellulose via cellulosome [6]. Some monosaccharides are used as a carbon source for growth by microorganisms, so the consumption of a high-fiber diet helps to increase the diversity of gut microbiota [7] and fiber-degradation bacteria such as *Prevotella* [8]. In contrast, a lack of dietary fiber will decrease microbial diversity [9], [10] and reduce the thickness of the mucus layer [11].

Short-chain fatty acids (SCFAs) are main fermentation products of dietary fibers by gut microbiota. The cecum is the principal place for microbial fermentation in chickens. A part of monosaccharides are fermented into SCFAs mainly including acetate, propionate and butyrate by SCFA-producing bacteria. *Bifidobacterium* produces acetate using bifid-shunt [12]. *Propionibacterium* produces propionate via a succinate-propionate pathway [13]. *Faecalibacterium* and *Roseburia* can use acetate to generate butyrate by butyryl-CoA: acetyl-CoA transferase [14]. SCFAs can serve as an important energy source [15]. Acetate is the main way for the host to obtain energy from dietary fiber. It can provide 1.2%–10% of the total energy per day for human beings. Propionate synthesizes glycogen in the liver. Butyrate provides energy for normal colonic epithelial cells [16] and promotes their proliferation [17]. SCFAs can increase the levels of intestinal peptides glucagon-like peptide-1 (GLP-1) and peptide tyrosine–tyrosine (PYY), thus reduce the appetite and energy intake of the host [18]. Propionate and butyrate activate intestinal gluconeogenesis (IGN) through a complementary mechanism to maintain glucose homeostasis [19]. SCFAs regulate the host metabolism, including energy metabolism, appetite regulation [20], glucose homeostasis and liver metabolism [21], which generally depend on specific G protein-coupled receptor (GPR) 43 (GPR43, also known as free fatty acid receptor 2 (FFAR2)) and GPR41 (FFAR3) [22]. GPR41 and GPR43 have been identified in humans [22], [23], mice [19], pigs and cattle, et al; however, the gene sequence of the SCFA receptors of chickens have not been included in the National Coalition Building Institute (NCBI) database, and related research reports are lacking.

The eubiotic lignocellulose OptiCell is a type of useful fiber. Its proportion of total dietary fiber is as high as 85%. So, no great adjustment was required in terms of the composition of the feed, generally adding 1.0%–1.5% can positively affect the growth performance and laying performance of poultry [24]. Results of experiments showed that the average daily gain and final weights of broilers increased by 9% and 7.8%, respectively, after adding 1% eubiotic lignocellulose for 32 days [25]. In addition, it is a synergistic combination of soluble and insoluble fiber, so it can produce more lactate and butyrate than traditional non-fermentable fiber *in vitro*. Due to the lack of enzymes encoding degradation fiber in the genome of chickens, gut microorganisms are responsible for degrading the dietary fiber in feed. Therefore, we speculated that the effects of eubiotic lignocellulose on chickens should be partly related to the gut microbiota. However, there were few reports on impacts of it on gut microbiota or metabolism of chickens.

Given this, we added different levels of eubiotic lignocellulose to the feed of chickens for 0–8 weeks, with the aim of observing the effects of eubiotic lignocellulose on the gut microbiota and microbial metabolite SCFAs and their receptors; we also aimed aiming to observe their affects on the growth performance, the development of the cecum and metabolism including appetite regulation, glucose homeostasis and the liver metabolism of chickens. Increasing our understanding of this would be beneficial to provide a theoretical basis for the application of eubiotic lignocellulose and improve the growth performance and health of chickens.

## **2. Materials And Methods**

### **2.1. Animals and Experimental Design**

This experiment was approved by the Shanxi Agricultural University Animal Experiment Ethics Committee, and the license number was SXAU-EAW-2017-002Chi.001. A total of 108 one-day-old ISA brown hens (IBH) with a 40 g average weight were chosen. Chickens were randomly divided into three groups, each group had 6 cages with 6 chickens per cage. According to the actual production, different levels of eubiotic lignocellulose OptiCell (OC) were added to the basic feed (Jinzhong Shiyang Feed Ltd, Shanxi, China) (Table 1) for 0-8 weeks. Group one was given 1% eubiotic lignocellulose and was called the OC-low (OL) group. Group two was given 2% eubiotic lignocellulose and was called the OC-high (OH) group. The control group was not given it, it was OC-free (OF) group. Samples were harvested to measure the gut microbiota, the concentration of SCFAs and so on of IBH at the end of 8 weeks.

The eubiotic lignocellulose (Beijing e-feed & e-vet cooperation, Beijing, China ) was developed by Agromed Ltd. (Austria), and it is made from special fresh timber. The composition of it ccontains energy ~0%, moisture 8%, crude protein 0.9%, total dietary fiber (TDF) 88%, crude ash 1.0%, crude fat 0.8%, minerals & trace elements 1.3%, crude fiber 59%, soluble TDF 1.3%, NDF 78%, ADF 64% and lignin 25%–30%.

Table 1  
Ingredients and nutrition level of feed during 0-8 weeks

| Ingredients (%)           |       | Nutrition level   |       |
|---------------------------|-------|-------------------|-------|
| Corn                      | 61.95 | ME (MJ/kg)        | 12.43 |
| Soybean meal              | 23.7  | Crude protein (%) | 19.49 |
| Soybean oil               | 1.1   | Crude fiber (%)   | 3.21  |
| Corn gluten meal          | 4     | Crude fat (%)     | 4.27  |
| DDGS                      | 4     | Crude ash (%)     | 5.83  |
| Stone power               | 1.8   | Ca (%)            | 1.05  |
| CaHPO <sub>4</sub>        | 1.3   | Total P (%)       | 0.57  |
| NaCl                      | 0.3   | NaCl (%)          | 0.3   |
| Met                       | 0.2   |                   |       |
| Lys                       | 0.46  |                   |       |
| Thr                       | 0.09  |                   |       |
| Multivitamin <sup>1</sup> | 0.4   |                   |       |
| Minerals <sup>2</sup>     | 0.55  |                   |       |
| Choline chloride          | 0.1   |                   |       |
| Complex enzyme            | 0.05  |                   |       |
| Total                     | 100   |                   |       |

<sup>1</sup> Feed (per kg) contains: vitamin A 2100-2500 KIU, vitamin D<sub>3</sub> 800-1240 KIU, vitamin E ≥ 5900 IU, vitamin K<sub>3</sub> ≥ 600 mg, vitamin B<sub>1</sub> ≥ 620 mg, vitamin B<sub>2</sub> ≥ 1600 mg, vitamin B<sub>6</sub> ≥ 830 mg, niacinamide ≥ 7000 mg, vitamin B<sub>12</sub> ≥ 4200 µg, pantothenic acid ≥ 2450 mg, folate ≥ 245 mg, biotin ≥ 35 mg. <sup>2</sup> Feed (per kg) contains: Cu 8 mg, Fe 80 mg, Mn 60 mg, Se 0.15 mg, Zn 40 mg, I 0.35 mg.

## 2.2. Management

Chickens were fed in brood cages for 0-8 weeks. Chickens were given free access to water and feed. The management of the temperature, light, and humidity was conducted according to the breeding manual of IBH. No conventional immunization schedule of chickens was performed to avoid impacts on gut microbiota. Body weights and feed intake of each group of chickens were recorded.

## 2.3. Sampling

We collected a part of feed and chose six chickens per group to collect the total feces for the determination of fiber digestibility. The blood was collected from the wing vein after these chickens were fasted for 12 h. The blood tube vessels were bathed in water at 37°C for 1 h, followed by 3000 r centrifugation for 10-15 min. The upper serum was absorbed into several 0.5 ml centrifuge tubes before being stored at -20°C until further blood glucose, GLP-1 and PYY analysis. They were executed with humanitarian slaughter and the length and weight of the cecum were measured. Several pieces of liver were also collected and preserved at -20°C until the determination of the content of liver glycogen. We sampled two pieces from the middle of the cecum and put them into 4% paraformaldehyde fixative solution for 24 h. The contents of the cecum were collected into multiple cryogenic tubes, and they were put into a liquid nitrogen tank and preserved at -80°C until the determination of the 16S rRNA gene sequence of gut microbiota and SCFAs. As above, the cecum was gathered to determine the relative expression of GPR43 mRNA.

## 2.4. Determination

### 2.4.1. 16S rRNA Gene Sequencing

The 16S rRNA gene of gut microbiota was sequenced by Genedenovo Biotechnology Ltd (Guangzhou, China) using High-Throughput Sequencing Technology. First, DNA extraction and PCR amplification were performed using the HiPure Stool DNA Kits (Magen, Guangzhou, China). V3-V4 regions of the 16S rRNA gene were amplified by PCR using primers 341F 5'-CCTACGGGNGGCWGCAG and 806R 3'-GGACTACHVGGGTATCTAAT. Illumina Hiseq 2500 sequencing was successively extracted.

Bioinformatics analysis. (1) Quality control and reads assembly. (2) OTU cluster. Effective tags were clustered into operational taxonomic units (OTUs) of  $\geq 97\%$  similarity using the UPARSE pipeline [26]. (3) Taxonomy classification. The representative sequences were classified into organisms by a Naive Bayesian Model using RDP classifier [27] based on SILVA Database [28]. Bacteria biomarker features of each group were screened by Metastats [29] and LEfSe (linear discriminant analysis (LDA) effect size) software [30]. Metastats showed significantly different bacteria using  $p < 0.01$  or 0.05. The value of LDA of certain microbes  $>2$  represents that the difference is significant. (4) Alpha diversity analysis. Alpha diversity indexes including ACE, Chao1, Shannon and Simpson were calculated in QIIME. ACE and Chao1 reflect the community richness, and Shannon and Simpson indices reflect the community richness and community diversity.

### 2.4.2. Crude Fiber Digestibility

The contents of crude fiber in the feed and manure were determined by the conventional method. Crude fiber digestibility (%) was calculated.

### **2.4.3. The Concentration of SCFAs**

The concentration of SCFAs (mmol/100g) in the cecum chyme was measured using the internal standard method with High Performance Gas Chromatography (HPGC) (Trace 1300, Thermo Fisher Scientific, America) [31].

### **2.4.4 Growth Performance**

The average daily feed intake per chicken per group was calculated as the average daily consumption divided by the number of chickens. The average daily gain of per chicken per group was calculated as follows: (current weight - previous weight) ÷ interval days ÷ number of chickens.

### **2.4.5. The Development of the Cecum**

The length and weight of the cecum were measured. Moreover, post 4% paraformaldehyde fixation, the cecum samples were processed using the conventional methods for tissue section, including washing, dehydration, transparency, embedding, slicing, deparaffinization and so on. The height of the cecum fold and the villous height on the cecum fold were measured.

### **2.4.6. Relative Expression of GPR43 mRNA**

We determined the relative expression of GPR43 mRNA using quantitative real-time PCR (qPCR). (1) Regarding the design and synthesis of primers. At present, the gene sequences of the SCFA receptors GPR43 (FFAR2) and GPR41 (FFAR3) of chicken have not been included in the NCBI database. Only the primers of GPR43 mRNA were designed and synthesized in this experiment. We found the uniformity of the coding sequences (CDSs) of GPR43 mRNA in humans, mice, cattle and pigs included in NCBI database was 89.7% using the DNAMAN software through BLAST (Figure 1). The conserved region of the highly homologous DNA sequence was selected (red area). Primers of GPR43 were designed with Primer Premier 3.0 software. Reference gene was  $\beta$ -actin. The primers were synthesized by Beijing Genomics Institute (BGI) (Shenzhen, Guangdong, China) (Table 2).

Table 2  
The design and synthesis of primers

|         | <b>Primer</b> | <b>Sequence</b>       |
|---------|---------------|-----------------------|
| GRP43   | Left primer   | TAGAACGCTACCTGGGAGTG  |
|         | Right primer  | ACCAGAGCAGCGATCACTC   |
| β-actin | Left primer   | GAGAAATTGTGCGTGACATCA |
|         | Right primer  | CCTGAACCTCTCATTGCCA   |

(2) For the extraction of total RNA, liver and cecum tissues were ground to powder with liquid nitrogen, and then 1 g of powder sample was put into a new 1.5 mL centrifuge tube and supplemented with 1 mL of RNAiso Plus (Thermo Fisher Scientific, America). The total RNA was then extracted. (3) For RNA detection, 2 µL of RNA sample was taken to detect the purity and concentration by a nucleic acid protein analyzer. The range of the good-quality OD260/OD280 (R value) should be 1.8–2.2. (4) For the first strand cDNA synthesis, the first strand of cDNA was synthesized by reverse transcription according to the instructions of Primescript™ RT Reagent Kit with gDNA Eraser (Perfect Real Time) (Takara Bio, Japan). The steps were as follows: first, we removed genomic DNA (Table 3a), with the following reaction procedure: 42°C for 2 min, 4°C for ∞. Second, we instigated the reverse transcription reaction (Table 3b), with the following reaction procedure: 37°C for 15 min, 85°C for 15 s, 4°C for ∞. (5) For the qPCR reaction system, GPR43 mRNA was amplified with corresponding primers using the cDNA of the cecum and liver tissues as a template. The qPCR reaction system was as follows (Table 3c). The qPCR reaction conditions were optimized, and the reaction conditions were as follows: pre denaturation at 95°C for 30 s, denaturation at 95°C for 5 s, 56°C for 30 s, 95°C for 15 s, 56°C for 1 min, 95°C for 30 s, 56°C for 15 s, 40 cycles.



Table 3  
Reaction system

| Reagent                                       | Dosage      |
|---|-------------|
| (a) DNA remove reaction system                |             |
| 5×gDNA Eraser Buffer                          | 2 μL        |
| gDNA Eraser                                   | 1 μL        |
| Total RNA                                     | 1 μg        |
| RNase Free dH <sub>2</sub> O                  | up to 10 μL |
| (b) The reverse transcription reaction system |             |
| 5×PrimeScript Buffer 2                        | 4 μL        |
| PrimeScript RT Enzyme Mix 1                   | 1 μL        |
| RT Prime Mix                                  | 1 μL        |
| RNase Free dH <sub>2</sub> O                  | 4 μL        |
| Reaction liquid (Table 2)                     | 10 μL       |
| (c) The qPCR reaction system                  |             |
| 2×Es Taq Master Mix                           | 10 μL       |
| Forward Primer, 10 μM                         | 1 μL        |
| Reverse Primer, 10 μM                         | 1 μL        |
| cDNA template                                 | 2 μL        |
| RNase Free dH <sub>2</sub> O                  | 6 μL        |

(6) Regarding the calculation method, first, the cycle threshold (CT) of the reference gene was normalized to the CT value of the target gene:  $\Delta CT$  (treatment groups) = CT (experiment target gene) - CT (experimental reference gene);  $\Delta CT$  (control group) = CT (control target gene) - CT (control reference gene). The target gene was GPR43, and the reference gene was  $\beta$ -actin. Second, the  $\Delta CT$  value of the control group was normalized to the  $\Delta CT$  value of the experimental groups:  $\Delta\Delta CT$  =  $\Delta CT$  (experiment groups) -  $\Delta CT$  (control group). Finally, the expression level ratio  $2^{-\Delta\Delta CT}$  was calculated. The value of the relative expression of GPR43 mRNA in the control group was regarded as 1, and the fold between the experiment groups and the control group was calculated.

## 2.4.7. Blood Glucose and Liver Glycogen

We determined the concentration of blood glucose (mmol/L) in the serum according to the instructions of Blood Glucose Kit (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China). We determined the content of liver glycogen (mg/g) according to the instructions of Liver Glycogen Kit (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China).

## 2.4.8. GLP-1 and PYY

We assayed the concentration of GLP-1 and PYY in the serum by using the Chicken GLP-1 ELISA kit and PYY ELISA kit (Shanghai Huyu Biotechnology Co., Ltd. Shanghai, China), respectively. The units of them are both pmol/L.

## 2.5. Statistical Analysis

In terms of gut microbiota, abundance statistics of each taxonomy were visualized using Krona [32]. The comparison of alpha diversity indexes between groups was calculated by Welch's t-test and Wilcoxon rank test using Vegan package in R project, and the comparison among groups was computed by Tukey's HSD test and the Kruskal-Wallis H test using Vegan package in R project [33]. Statistical analyses of other indexes were performed using a one-way analysis of variance (ANOVA) with Statistical Product and Service Solutions (SPSS) 22.0 (IBM). The results were expressed as the means and standard error of the mean (SEM).

## 3. Results

### 3.1. The Determination of Gut Microbial Diversity and Composition of IBHE

#### 3.1.1. OTUs and Gut Microbial Diversity of IBHE

IBHE refer to "ISA Brown Hens-eight weeks". IBHE contained the OC-low (OL) group, OC-high (OH) group and OC-free (OF) group.

The total and unique numbers of OTUs in OL, OH and OF group were similar (Figure 2). This was consistent with the lack of a significant difference ( $P > 0.05$ ) among groups of IBHE in terms of  $\alpha$ -diversity (Table 4). It indicated that added eubiotic lignocellulose had little effect on the gut microbial diversity of chickens.

**Figure 2** Venn diagram of operational taxonomic units (OTUs)

Note: The overlapping parts show the OTUs shared by three groups, and the numbers on both sides are the unique OTUs owned by each group.

Table 4  
Comparison of  $\alpha$ -diversity of gut microbiota among groups of IBHE

|                  | IBRE-OL | IBRE-OH | IBRE-OF | SEM   | P-value |
|------------------|---------|---------|---------|-------|---------|
| ACE              | 1518.33 | 1572.97 | 1594.39 | 70.17 | 0.14    |
| Chao1            | 1522.07 | 1543.41 | 1547.78 | 71.80 | 0.79    |
| Observed_species | 1109.67 | 1109.67 | 1114.33 | 54.60 | 0.73    |
| Shannon          | 6.87    | 6.64    | 6.54    | 0.37  | 0.67    |
| Simpson          | 0.97    | 0.96    | 0.95    | 0.018 | 0.95    |

### 3.1.2. Gut Microbial Composition of IBHE

Dietary fiber including the eubiotic lignocellulose is broken down into monosaccharide by fiber-degradation bacteria [2]. A part of monosaccharides are then fermented into SCFAs by SCFA-producing bacteria. Therefore, we focused on significantly different fiber-degradation bacteria and SCFAs-producing bacteria between groups.

Compared with the OH and OF groups, the dominant bacteria of the OL group were the butyrate-producing bacterium *Oscillospira* (Figure 3a) and the lactate-producing bacterium *Lactobacillus panis* (Figure 4a). Compared with the OF group, OL also had more of a low abundance of the fiber-degradation bacteria *Prevotella*, *Roseburia* and the lactate-producing bacterium *Lactobacillus oris* (Figure 3b). *Roseburia* is also a butyrate-producing bacteria. Compared with the OL and OF groups, the dominant bacteria of the OH group had the fiber-degradation bacterial species *Bacteroides thetaiotaomicron*. *B. thetaiotaomicron* is one of the most excellent fiber-degradation bacteria. Moreover, compared with the OF group, OL and OH groups both had *B. thetaiotaomicron* and the acetate-producing bacterium *Sutterella* (Figure 3a-c; 4b,c). Notably, *B. thetaiotaomicron* was lacking in the OF group (Figure 4b).

(a) IBHE-OH vs. IBHE-OL (b) IBHE-OF vs. IBHE-OL (c) IBHE-OF vs. BHE-OH

**Figure 3** LDA (linear discriminant analysis) between the groups of IBHE

(a) Abundance histogram of dominant bacterium *Lactobacillus panis* in IBHE-OL

(b) Abundance histograms of the dominant fiber-degradation bacterium *B. thetaiotaomicron* and (c) the acetate-producing bacterium *Sutterella* in IBHE-OL and IBHE-OH

**Figure 4** Abundance histogram of some dominant bacteria among three groups of IBHE

## 3.2. Crude Fiber Digestibility

The results showed that the crude fiber digestibility of OL and OH groups was significantly higher ( $P < 0.05$  and  $0.01$ ) than the OF group (Figure 5). This was consistent with the fact that OL and OH groups had fiber-degrading bacterium *B. thetaiotaomicron*, while it was lack in the OF group.

**Figure 5** Histogram of crude fiber digestibility among three groups of IBHE

Note: Means within a line lacking a common lowercase superscript letter mean significant differences with a  $P$ -value  $< 0.05$ , and means within a row lacking a common uppercase superscript letter means extremely significant differences with a  $P$ -value  $< 0.01$ .

### 3.3. Concentration of SCFAs in IBHE

SCFAs are main fermentation products of dietary fibres by gut microbiota. So we next observed the effects of the difference of the gut micriobiota among groups on the concentration of SCFAs. The concentration of acetate, propionate and butyrate in the OL group was significantly higher ( $P < 0.01$  or  $0.05$ ) than the other two groups, indicating that the addition of 1% eubiotic lignocellulose was more conducive to the production of SCFAs at 8 weeks (Table 5).

Table 5  
Comparison of the concentration of short-chain fatty acids (SCFAs) (mmol/100g) among groups of ISA brown hens (IBHE)

| SCFA       | IBHE-OL            | IBHE-OH             | IBHE-OF            | SEM   | <i>P</i> -value |
|------------|--------------------|---------------------|--------------------|-------|-----------------|
| Acetate    | 4.07 <sup>Aa</sup> | 3.01 <sup>Bb</sup>  | 2.42 <sup>Bb</sup> | 0.25  | 0.00            |
| Propionate | 0.96 <sup>Aa</sup> | 0.72 <sup>ABb</sup> | 0.62 <sup>Bb</sup> | 0.077 | 0.01            |
| Butyrate   | 0.28 <sup>a</sup>  | 0.20 <sup>b</sup>   | 0.19 <sup>b</sup>  | 0.026 | 0.027           |

Means within a line lacking a common lowercase superscript letter mean significant differences with a  $P$ -value  $< 0.05$ , and means within a row lacking a common uppercase superscript letter mean extremely significant differences with a  $P$ -value  $< 0.01$ .

### 3.4. Growth Performance

Given the concentration of SCFAs in the OL group was higher than the other two groups (Table 5) and SCFAs can provide energy for the host, we next determined the growth performance of chickens. There was no significant difference ( $P > 0.05$ ) among groups in terms of the on the body weight (BW), average daily feed intake (ADF) and average daily gain (ADG) of ISA brown hens (IBHE) growth performance (Table 6).

Table 6  
Effects of eubiotic lignocellulose on the growth performance of ISA brown hens (IBHE)

|         | IBHE-OL | IBHE-OH | IBHE-OF | SEM   | <i>P</i> -value |
|---------|---------|---------|---------|-------|-----------------|
| BW (g)  | 775.50  | 764.38  | 766.25  | 15.44 | 0.25            |
| ADF (g) | 52.39   | 51.62   | 50.59   | 1.04  | 0.91            |
| ADG (g) | 13.85   | 13.16   | 13.73   | 2.25  | 0.36            |

### 3.5. The Development and Histomorphology of the Cecum

Because the concentration of butyrate in the OL group was the highest (Table 5) and butyrate can provide ~70% energy for normal intestinal epithelial cells and promotes their proliferation [17]; therefore, we next determined the development and histomorphology of the cecum. However, there was no significant difference ( $P > 0.05$ ) among groups in terms of the development (Table 7) and histomorphology of the cecum of chickens (Figure 6).

Table 7  
Effects of eubiotic lignocellulose on the development and histomorphology of the cecum

|                                | IBHE-OL | IBHE-OH | IBHE-OF | SEM   | <i>P</i> -value |
|--------------------------------|---------|---------|---------|-------|-----------------|
| Cecum                          |         |         |         |       |                 |
| Length (cm)                    | 12.1    | 12.3    | 12.03   | 0.9   | 0.91            |
| Total weight (g)               | 3.31    | 2.94    | 2.77    | 0.41  | 0.16            |
| Chyle weight (g)               | 0.99    | 0.94    | 0.76    | 0.35  | 0.36            |
| Caecum fold                    |         |         |         |       |                 |
| Height ( $\mu\text{m}$ )       | 479.57  | 525.51  | 437.3   | 36.13 | 0.29            |
| Villi height ( $\mu\text{m}$ ) | 153.91  | 148.09  | 152.77  | 16.07 | 0.96            |

(a) IBHE-OL                      (b) IBHE-OH                      (c) IBHE-OF

**Figure 6** Histological section of the cecum among groups of IBHE

Note: Blue arrows show the heights of cecum folds and yellow arrows show the heights of villi on the cecum fold

### 3.6. Relative Expression of GPR43 mRNA in IBHE

The SCFAs are received by the specific receptors such as GPR43 on the cell surface [22], thus initiating the intracellular signal transduction pathway, regulating the metabolism of the host. Therefore, we next determined the relative expression of GPR43 mRNA of chickens.

In this study, the range of CT values of cecum target gene GPR43 was 23–24, and that of the liver was 21–23. The CT value of cecum reference gene  $\beta$ -actin was 16–18, and that of liver was 18–19. The results showed that GPR43 mRNA was expressed well and the primer of it could be applied to chickens.

There was no significant difference ( $P > 0.05$ ) among groups in the relative expression of GPR43 mRNA in the cecum and liver, but it was higher in OL and OH groups, which both had added eubiotic lignocellulose, than in the OF group (Figure 7).

**Figure 7** Histogram of the expression of G protein-coupled receptor 43 (GPR43) mRNA among three groups of ISA brown hens (IBHE)

### 3.7. Concentration of GLP-1 and PYY

The SCFA receptors GPR41 or GPR43 can induce the release of GLP-1 and PYY, making the host feel satiety and reducing appetite [34]. So we next determined the concentration of GLP-1 and PYY.

There was no significant difference ( $P > 0.05$ ) among groups in the concentration of intestinal peptides GLP-1 and PYY (Figure 8), indicating that added eubiotic lignocellulose had little effect on the appetite of the host. This was consistent with there being no significant difference ( $P > 0.05$ ) in the average daily feed intake (ADF) (Table 6) and relative expression of GPR43 mRNA (Figure 7).

(a) Histogram of GLP-1                      (b) Histogram of PYY

**Figure 8** Histograms of the concentration of glucagon-like peptide-1 (GLP-1) and peptide tyrosine-tyrosine (PYY) among three groups of ISA brown hens (IBHE)

### 3.8. Blood Glucose and Liver Glycogen

SCFAs also regulate the host metabolism, including glucose homeostasis and liver metabolism [21], which generally depend on specific SCFA receptors [22]. We next determined the blood glucose and liver glycogen of chickens.

The added eubiotic lignocellulose had no significant effect ( $P > 0.05$ ) on the blood glucose and liver glycogen of chickens (Figure 9). This was consistent with there being no significant difference ( $P > 0.05$ ) in the relative expression of GPR43 mRNA (Figure 7).

**Figure 9** Histogram of content of blood glucose and liver glycogen among three groups of ISA brown hens (IBHE)

## 4. Discussion

### 4.1. Effects of Eubiotic Lignocellulose on Fiber-Degradation Bacteria and Fiber Digestibility

Dietary fiber is the substrate of fiber-degradation bacteria and it is broken down into monosaccharide by them [2]. The consumption of a high-fiber diet helps to increase the richness and diversity of gut microbiota [7]. Excellent fiber-degradation genera include *Bacteroides*, *Prevotella*, *Ruminococcus*, *Fibrobacter* and *Roseburia* [35]. Excellent fiber-degradation species include *Bacteroides thetaiotaomicron* [36], *Bacteroides ovatus* [3], *Ruminococcus flavefaciens* and so on.

In this experiment, the addition of eubiotic lignocellulose did not increase microbial diversity, but increased the relative abundance of *B. thetaiotaomicron*, which is one of the most excellent fiber-degradation bacteria. *B. thetaiotaomicron* can motivate more than a quarter of the genome to degrade polysaccharides such as cellulose into monosaccharides [36] by CAZymes which were encoded by polysaccharide utilization locus (PUL) [37]. PUL has been identified in all members of Bacteroidetes such as *B. thetaiotaomicron*, and about 18% of its genome is attributed to these clusters. PULs of *B. thetaiotaomicron* encode twenty-five different CAZymes to degrade rhamnogalacturonan II (RG-II) which is known to be the most complex glycan in pectin [38]. *B. thetaiotaimicron* also extracellularly releases outer membrane vesicles (OMVs) containing glucohydrolase to remotely degrade dietary fiber [39]. Therefore, the crude fiber digestibility of OL and OH groups with added eubiotic lignocellulose was shown to be significantly higher than the OF group in this study.

Moreover, compared with the OF group, the dominant bacteria of OL group had a low abundance of the excellent fiber-degradation bacteria *Prevotella* and *Roseburia*. It was reported that *Prevotella*, *Xylanibacter* and *Treponema* were unique in African children with high-fiber diets compared with Italian children with low-fiber diets, and these bacteria contained genes for degrading cellulose and xylan, which could maximize the harvest of energy from plant polysaccharides [8]. *Roseburia intestinalis* is an excellent xylan-degrading species [35]. Our results suggested that the addition of 1% eubiotic lignocellulose is beneficial for increasing the abundance of certain fiber-degradation bacteria at 8 weeks.

### 4.2. Effects of Added Eubiotic Lignocellulose on SCFA-Producing Bacteria and SCFAs

Dietary fiber is cleaved into monosaccharides by fiber-degradation bacteria before it is fermented into SCFAs by SCFA-producing microorganisms. Acetate, propionate and butyrate account for 90%-95% of SCFAs. *Bifidobacterium*, *Sutterella* and *Blautia* are acetate-producing bacteria. Propionate-producing

bacteria include *Propionibacterium*, *Phascolarctobacterium* and *Veillonella* [13]. Butyrate-producing bacteria include *Faecalibacterium* [40], *Roseburia*, *Coproccus* [41], *Oscillospira*, *Anaerostipes* and so on. Some fiber-degrading bacteria such as *Bacteroides*, *Prevotella* and *Roseburia* are also SCFA-producing bacteria.

In this experiment, the relative abundances of the lactate-producing bacterium *Lactobacillus panis* and butyrate-producing bacterium *Oscillospira* in the OL group were higher than other groups. Moreover, compared with the OF group, the dominant bacteria of the OL group included the butyrate-producing such as *Roseburia*, *Oscillospira* and *Subdoligranulum* and lactate-producing bacteria *Lactobacillus oris* at 8 weeks. Thus, the concentration of acetate and butyrate in the OL group was the highest among the three groups. The eubiotic lignocellulose is a synergistic combination of soluble and insoluble fiber, so it can produce more lactate and butyrate than traditional non-fermentable fiber *in vitro* [25]. It suggested that the addition of 1% eubiotic lignocellulose was more conducive to increasing the abundance of certain SCFA-producing bacteria and the concentration of SCFAs at 8 weeks in this experiment.

### 4.3. Effects of Eubiotic Lignocellulose on Growth Performance

SCFAs, which are generated by the fermentation of dietary fiber by the gut microbiota can constitute an important energy source for the host [13]. *Bacteroides thetaiotaomicron* in the intestinal tract of mice can ferment cellopolysaccharide to produce SCFAs to provide energy for the host [36]. Acetate is the main way for the body to obtain energy from dietary fiber. The oxidation of acetate provides 0.876 MJ/mol of energy, which can provide 1.2%–10% of the total energy per day for human beings [42]. In this study, the effect of added eubiotic lignocellulose on the body weight and average daily gain (ADG) of chickens was not significant. One reason for this was that eubiotic lignocellulose itself has little energy; the other reason was that, although the concentration of SCFAs in the OL group was higher than other two groups, acetate only increased by 1.06–1.65 mmol/100g cecum chyme, and it did not provide much energy for the host. Therefore, it had little effect on the body weight and ADG of chickens.

Moreover, the feed intake of broilers increased by 6.7%–9.4%, after adding 1%–1.5% eubiotic lignocellulose [25]. However, in this study, added eubiotic lignocellulose had no significant effect on the average daily feed intake (ADF) of IBH. This was attributed to the fact that there was no difference in the gastrointestinal hormones GLP-1 and PYY. At first, it was believed that the satiety effect of dietary fiber for the host was mainly due to water absorption and the swelling effect; later, it was confirmed that this actually was due to the SCFAs increasing the levels of intestinal peptides such as GLP-1 and PYY secreted by intestinal endocrine L cells, and then increasing the activity of receptors acting on arcuate nucleus (ARC) and nucleus tractus solitarius (NTS) [43], thus reducing the appetite and food intake of the host [18].



## **4.4. Effects of Eubiotic Lignocellulose on the Development of the Cecum**

The cecum or colon is the principal place for the microbial transfer of dietary fiber in monogastric animals. Butyrate is the first choice of intestinal epithelial cells [44]. Studies have shown that about 95% of butyrate is absorbed into epithelial cells and is rapidly  $\beta$ -oxidized into ketones for ATP synthesis [45]. Butyrate provides ~70% energy for normal intestinal epithelial cells [45] and promotes their proliferation [17]. Compared with mice fed a fiber-free diet, the colon length of mice fed a fiber-rich diet or normal diet increased [46]. However, there was no significant difference in the development and histomorphology of the cecum in this experiment. This was because, although the butyrate content of the OL group was the highest, the difference between the three groups was not great. Therefore, there was little energy supply for cecum epithelial cells, and it was not sufficient to cause significant differences in intestinal morphology.

## **4.5. Effects of Eubiotic Lignocellulose on Relative Expression of GPR43 mRNA and Metabolism of Chickens**

### **4.5.1. Effects of eubiotic lignocellulose on GLP-1 and PYY**

At present, the gene sequences of the SCFA receptors GPR43 (FFAR2) and GPR41(FFAR3) of chickens have not been included in the NCBI database. In this study, the primer sequence and reaction conditions of GPR43 gene in this experiment can be applied to chicken. The SCFAs were received by the specific receptors GPR43 or GPR41 on the cell surface [22], thus initiating the intracellular signal transduction pathway, regulating the metabolism of the host. Studies have found that SCFAs can induce the release of GLP-1 and PYY via the recognition of receptors GPR41 or GPR43, making the host feel satiety and reducing food intake [46]. In this study, added eubiotic lignocellulose had no significant effect on the relative expression of GPR43 mRNA in the cecum and liver, resulting in there being no significant difference among groups in terms of the serum GLP-1 and PYY of chickens. Thus, there was no significant difference in ADF.

### **4.5.2. Effects of eubiotic lignocellulose on blood glucose and liver glycogen**

SCFAs can also regulate the glucose homeostasis of hosts through intestinal gluconeogenesis (IGN). Gluconeogenesis is beneficial to the glucose and energy balance. Most propionate and acetate are absorbed into the portal vein [47] and enter the liver, affecting glucose metabolism. Studies have shown that the SCFAs butyrate and propionate activate IGN via complementary mechanisms [19]. Butyrate activates IGN gene expression through a cyclic adenosine monophosphate (cAMP)-dependent mechanism, and the substrate of gluconeogenesis—propionate activates IGN gene expression via a gut–

brain neural circuit involving SCFA receptors [19]. In this study, there was no significant difference in blood glucose among groups, resulting in there was being no significant difference in blood glucose among groups.

Moreover, acetate and butyrate can increase the phosphorylation of AMP-activated protein kinase (AMPK) in the liver by increasing the ratio of AMP to ATP and upregulation of peroxisome proliferator-activated receptor alpha (PPAR $\alpha$ ) gene, thus increase liver glycogen storage, which is possibly mediated via a GPR41/GPR43-dependent mechanism [23]. In this study, added eubiotic lignocellulose had no significant effect on the relative expression of GPR43 mRNA, resulted in there was being no significant difference in terms of the liver glycogen among groups.

## 5. Conclusion

The addition of eubiotic lignocellulose has little effects on the growth performance and the development and histomorphology of the cecum of chickens, but can increase the abundance of the fiber-degradation bacterium *Bacteroides thetaiotaomicron* and the fiber digestion. The addition of 1% eubiotic lignocellulose is beneficial to the production of SCFAs at 8 weeks. The primer sequences of the GPR43 mRNA and reaction conditions designed in this experiment can be applied to chickens. The effects of added eubiotic lignocellulose on the relative expression of GPR43 mRNA were slight, resulting in the differences among groups in terms of the metabolism of chickens. The primers of GPR41 and GPR109A should be designed and synthesized in the future, to observe the effects of these SCFA reporters on the metabolism and health of chickens.

## Abbreviations

ABC: ATP-binding cassette; ADFI: average daily feed intake; ADG: average daily gain; AMP: adenosine monophosphate; ANOVA: analysis of variance; CAZymes: Carbohydrate-Active enZymes; CDS: coding sequence; CT: cycle threshold; FFAR: free fatty acid receptor; FFAR2: free fatty acid receptor 2; FFAR3: free fatty acid receptor 3; GLP-1: Glucagon-like peptide-1; gpPULs: gram-positive PULs; GPR: G protein-coupled receptor; GPR41: G protein-coupled receptor 41; GPR43: G protein-coupled receptor 43; IBH: ISA Brown Hens; IBHE: ISA Brown Hens-8 weeks; IGN: intestinal gluconeogenesis; LDA: linear discriminant analysis; OC: OptiCell; OL: OC-Low; OH: OC-High; OF: OC-Free; OTU: operational taxonomic unit; OMVs: outer membrane vesicles; PPAR: peroxisome proliferators-activated receptors; PUL: polysaccharide utilization locus; PYY: peptide tyrosine-tyrosine; RG-II: rhamnogalacturonan II; SCFA: short-chain fatty acid; SEM: standard error of the mean ; SPSS: Statistical Product and Service Solutions; TCS: two-component system; TDF: total dietary fiber.

## Declarations

## Ethics approval and consent to participate

This experiment was approved by the Shanxi Agricultural University Animal Experiment Ethics Committee, and the license number was SXAU-EAW-2017-002Chi.001.

## Consent for publication

All the authors read and agree to the content of this paper and its publication.

## Availability of data and material

All data generated or analysed during this study are included in this published article.

## Competing interests

The authors declare that they have no competing interests.

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## Authors' contributions

Conceptualization, Y.Y.; methodology, L.H.; formal analysis, B.S and L.H.; investigation, B.S. and L.H.; resources, Y.Y.; data curation, B.S. and L.H.; writing—original draft preparation, B.S.; writing—review and editing, B.S.; visualization, L.H.; supervision, Y.Y.; project administration, Y.Y. and B.S.; funding acquisition, Y.Y.

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# Figures

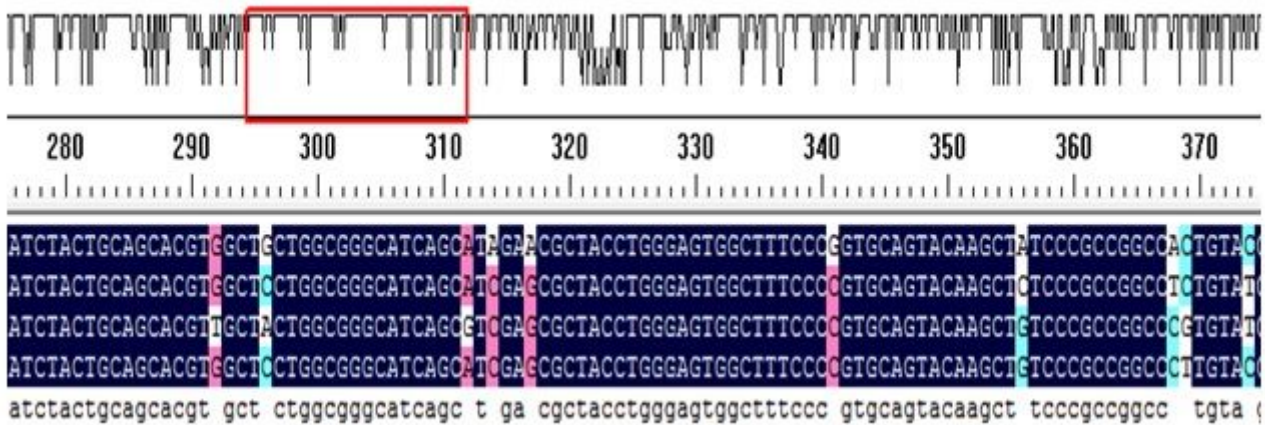


Figure 1

BLAST of coding sequences of G protein-coupled receptor 43 (GPR43) in humans, mice, cattle and pigs

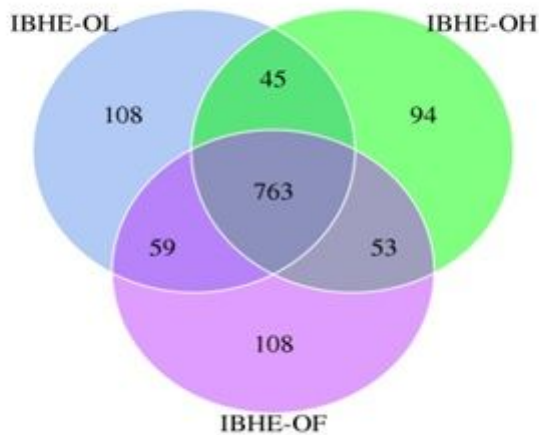
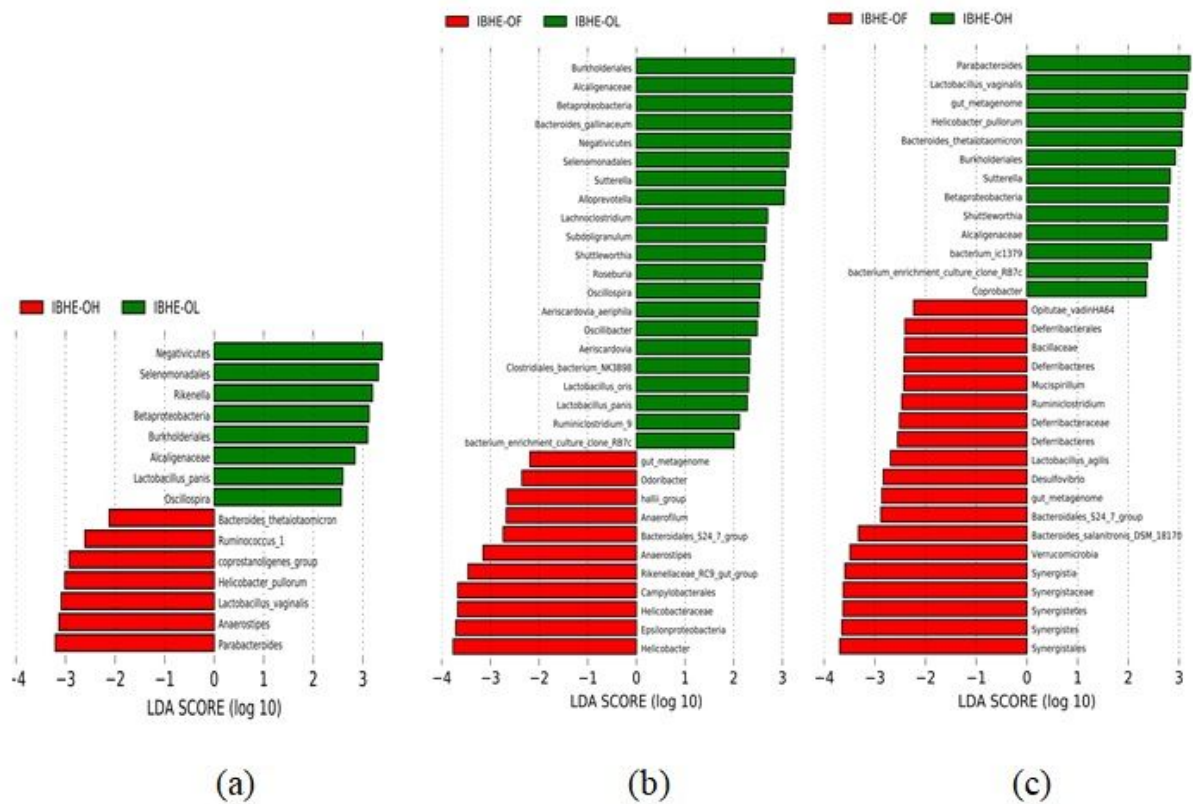


Figure 2

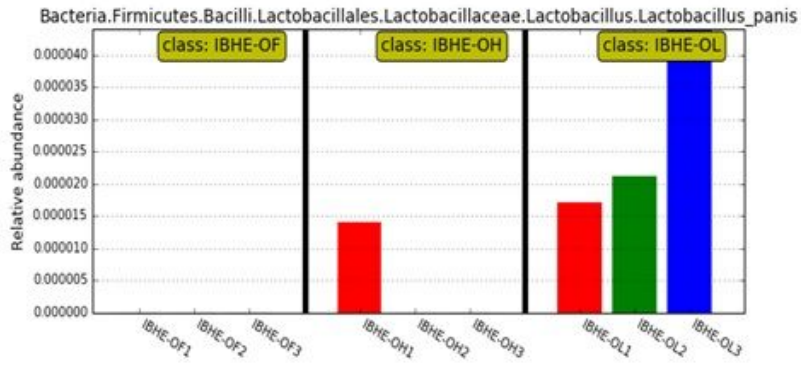
Venn diagram of operational taxonomic units (OTUs) Note: The overlapping parts show the OTUs shared by three groups, and the numbers on both sides are the unique OTUs owned by each group.



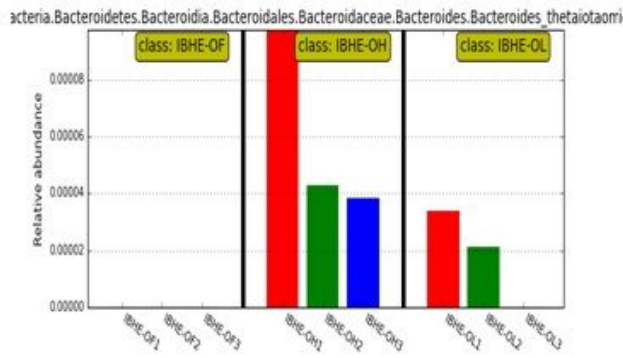
**Figure 3**

LDA (linear discriminant analysis) between the groups of IBHE (a) Abundance histogram of dominant bacterium *Lactobacillus panis* in IBHE-OL (b) Abundance histograms of the dominant fiber-degradation bacterium *B. thetaiotaomicron* and (c) the acetate-producing bacterium *Sutterella* in IBHE-OL and IBHE-OH

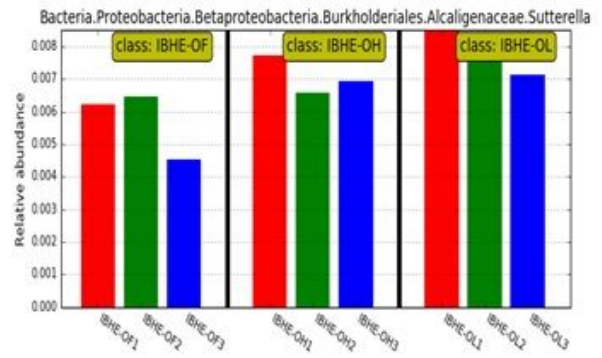




(a)



(b)



(c)

Figure 4

Abundance histogram of some dominant bacteria among three groups of IBHE

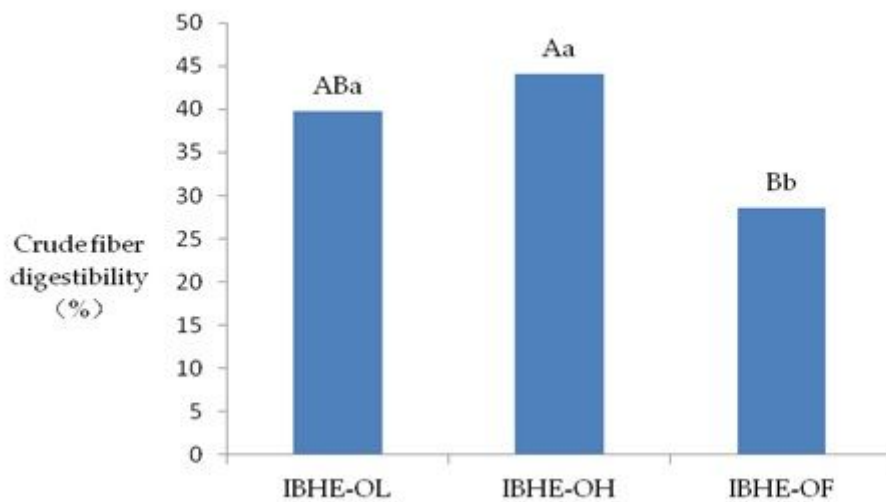
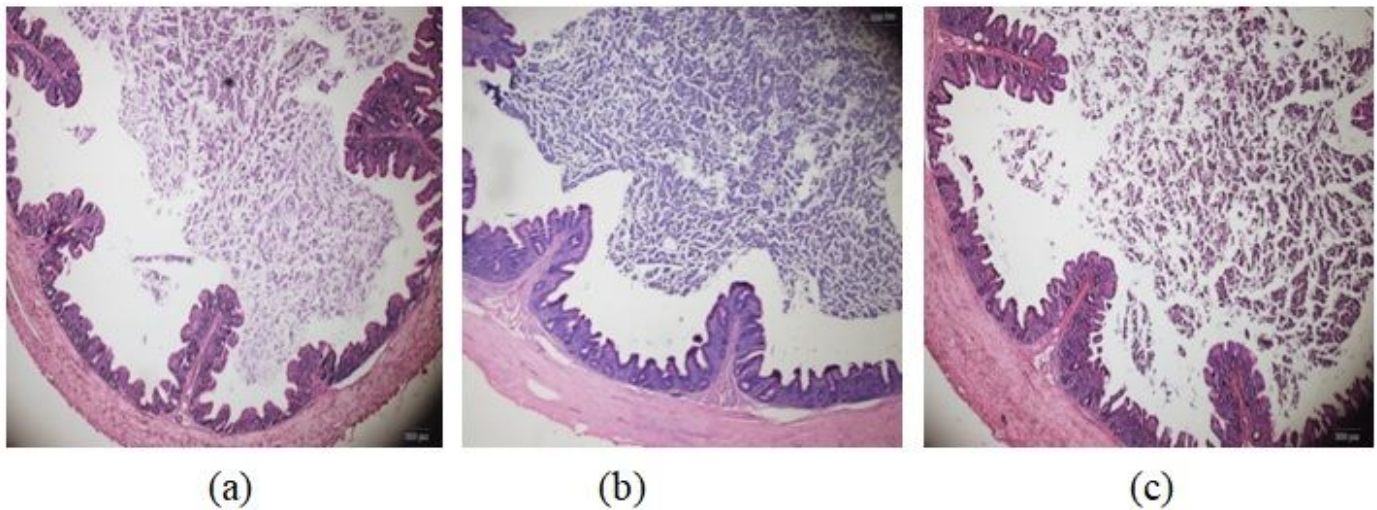


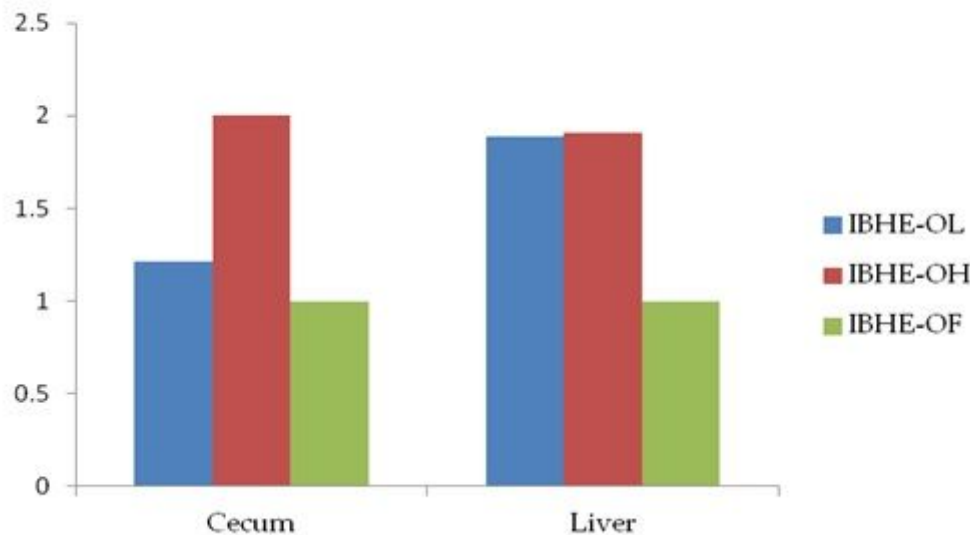
Figure 5

Histogram of crude fiber digestibility among three groups of IBHE Note: Means within a line lacking a common lowercase superscript letter mean significant differences with a P-value < 0.05, and means within a row lacking a common uppercase superscript letter means extremely significant differences with a P-value < 0.01.



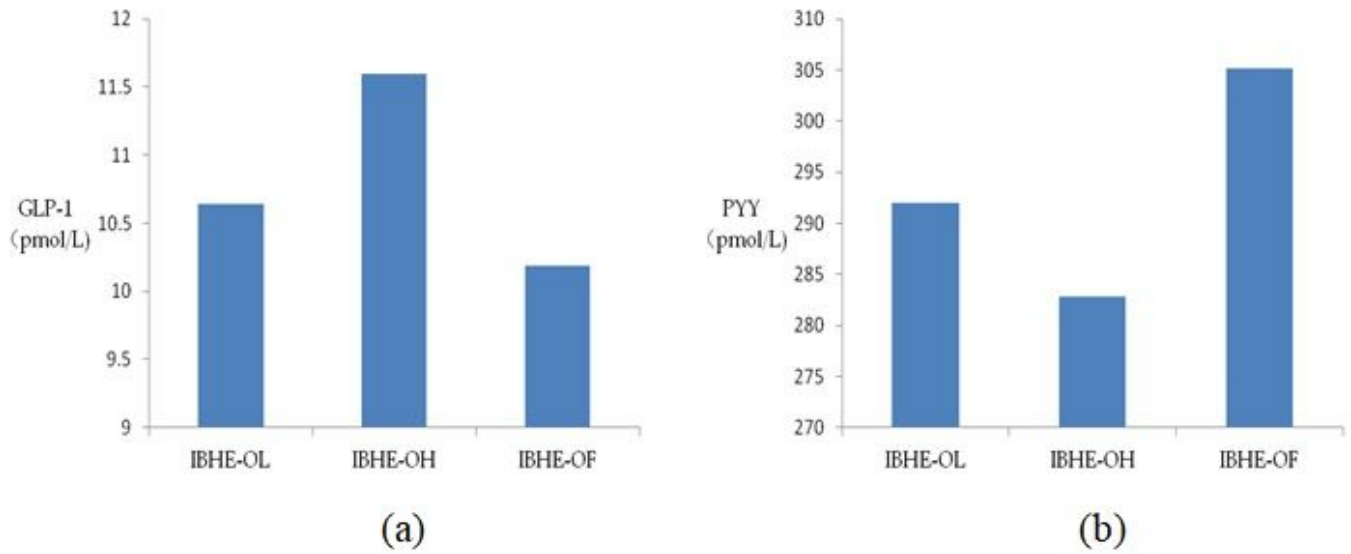
**Figure 6**

Histological section of the cecum among groups of IBHE Note: Blue arrows show the heights of cecum folds and yellow arrows show the heights of villi on the cecum fold (a) IBHE-OL (b) IBHE-OH (c) IBHE-OF



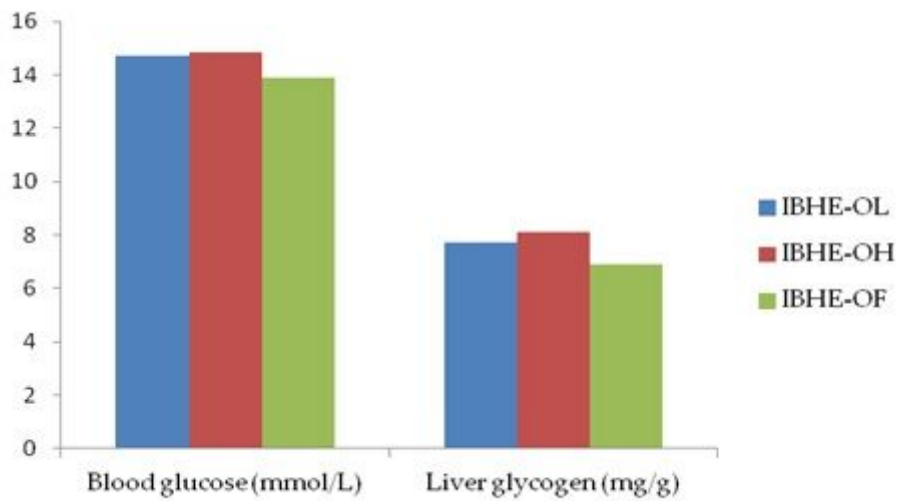
**Figure 7**

Histogram of the expression of G protein-coupled receptor 43 (GPR43) mRNA among three groups of ISA brown hens (IBHE)



**Figure 8**

Histograms of the concentration of glucagon-like peptide-1 (GLP-1) and peptide tyrosine-tyrosine (PYY) among three groups of ISA brown hens (IBHE)



**Figure 9**

Histogram of content of blood glucose and liver glycogen among three groups of ISA brown hens (IBHE)