

Article

An Appropriate Amount of OptiCell Benefits the Microbial Diversity and Gut Health of Chickens

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Simple Summary: In recent years, more and more research has focused on the effects of free-range rearing on the welfare of chickens. However, few works have focused on the gut microbial diversity and gut health of free-range chickens, especially when plant fibers are lacking in the wild. A lack of dietary fiber will decrease the microbial diversity and even damage the gut health of the host, so we added eubiotic lignocellulose OptiCell to the feed of caged and free-range Chinese local Bian chickens at three different levels (0%, 2%, 4%) from September to November, aiming to determine an appropriate amount of OptiCell for effectively improving the gut microbial diversity and gut health of chickens when available plant fibers are scarce. The results showed that adding 2% OptiCell was optimum for free-range chickens, while 4% OptiCell was optimum for caged chickens. In addition, compared with the 2% OptiCell in the cage mode, 2% OptiCell in free-range rearing could effectively improve unique microbial diversity and gut development, which was beneficial to the welfare and gut health of chickens who suffered from a lack of plant fibers in the wild.

Abstract: It is of merit to study the appropriate amount of fiber to add to free-range chickens feed to improve the microbial diversity and gut health in times of plant fiber deprivation. OptiCell is a useful source of fiber as a type of eubiotic lignocellulose, and its positive effects on the growth performance and laying performance of chickens has already been proven. However, few researchers have researched the effects of adding OptiCell on the gut microbiota of chickens. In this research we added three different levels of OptiCell (0%, 2% and 4%) to the feed of caged and free-range Bian chickens from September to November, aiming to observe the effects of adding OptiCell and different feeding modes on the gut microbial diversity and gut health of chickens, and aiming to determine an appropriate amount of OptiCell. The results showed that adding OptiCell could increase the thickness of the cecum mucus layer and the abundance of *Akkermansia* and *Faecalibacterium* in caged chickens, and 4% OptiCell was optimum. In addition, adding OptiCell increased the microbial diversity and the abundance of the butyrate-producing bacteria *Faecalibacterium* and *Roseburia* of free-range chickens. The α -diversity and the length of the small intestine with 2% OptiCell in free-range chickens were better than with 2% OptiCell in caged chickens. In addition, compared with caged chickens, the free-range chickens had longer small intestine and lower GLP-1. Taken together, an appropriate amount of OptiCell benefitted the microbial diversity and health of chickens; it was necessary to add dietary fiber to the feed of free-range chickens when plant fibers was lacking, and 2% OptiCell was found to be optimum.

Keywords: OptiCell; microbial diversity; gut microbiota; gut health; free-range chickens; caged chickens; SCFA; mucus layer; welfare

1. Introduction

In recent years, animal welfare has attracted more and more social attention. The “world farm animal welfare conference, ▪ Beijing consensus” was reached in Beijing in 2018, and the 3rd World Conference on Farm Animal Welfare (WCAFW) was held in Qingdao in 2019. In terms of chicken welfare, the European Union had completely abolished the traditional cage mode of laying hens by 2012, and Belgium plans to change to the free-range mode by 2024.

Free-range chickens can access natural environments and more space, and have more opportunities to show natural behaviors such as foraging and sand bathing [1]. Free-range rearing is not only beneficial to welfare [2,3], but is also beneficial to the gut microbial diversity of chickens. Compared with caged chickens, free-range chickens who forage various plants have more abundant gut microbiota [4,5] and more *Actinobacteria* [6]. Varied and healthy gut microbiota depends on the diversity of the diet [7].

However, the decrease of food source diversity and dietary fiber content can both reduce the gut microbial diversity of the host [8,9]. The gut microbiota of mice who were fed low fiber long-term lost diversity, and this was seriously compounded over generations [10]. The loss of gut microbiota will result in some diseases. For example, both obesity and type 2 diabetes have a common feature, which is the reduction of gut microbial diversity [11,13]. Therefore, gut microbial diversity has been a major focus of the Human Microbiome Project (HMP) [14,15]. In contrast, people who consume more dietary fiber have more gut microbiota [16,17], because dietary fiber is the substrate of bacteria and it can be degraded into glucose and other monosaccharides, which provide carbon and energy sources for the proliferation of other microorganisms [18]. Some of these monosaccharides can be fermented to short chain fatty acids (SCFAs) by microbiota, mainly including acetate, propionate and butyrate. Acetate is used as energy for peripheral tissues. Propionate produces glycogen and butyrate provides ~70% energy for normal colonic epithelial cells [19] and promotes their proliferation [20]. Butyrate-producing bacteria include *Faecalibacterium*, *Roseburia*, *Coproccus*, *Anaerostipes* and so on. *Faecalibacterium prausnitzii* [21] and *Roseburia intensis* [22] can use acetate to produce butyrate. Butyrate can increase the thickness of the mucus layer, preventing the invasion of pathogenic bacteria [23] and maintaining gut health. In addition, SCFAs can activate intestinal gluconeogenesis (IGN) [24,25] and induce the release of glucagon like peptide-1 (GLP-1) to make the host feel satiety and reduce food intake [26,27]. Therefore, suitable dietary fiber can improve the gut microbial diversity, the production of SCFAs, and the gut health of the host.

The Bian chicken is a kind of Chinese local chicken, which is suitable for free-ranging. However, the available plant fiber reduces over time while chickens are free-ranging especially from autumn to winter, and a lack of plant fiber will decrease the microbial diversity and gut health of the chickens. Therefore it is necessary to add dietary fiber to the feed of free-range chickens when they suffer from plant fiber deprivation. However, doing so is not completely safe, as all kinds of dietary fiber, such as the soluble dietary fiber inulin, has been shown to induce cancer in mice with disordered gut microbiota [28]. This highlights the importance of choosing the right kind of fiber, in this case OptiCell, for poultry. OptiCell® was developed by Agromed Ltd.(Austria), and is a safe eubiotic lignocellulosic product which is made from fresh timber. Its crude fiber content is as high as 59% [29], so generally adding 1.0% ~ 1.5% can positively affect the feed intake and growth performance of poultry [30]. In addition, OptiCell has an excellent ratio of soluble and insoluble fiber, so can produce more lactate and butyrate than traditional non fermentable fiber in vitro. OptiCell also absorbs pathogens and improves intestinal health. China began to import it in 2012. However, it was not widely used in China at first and there are few reports on the effects of OptiCell on the gut microbiota of chickens in China. Given this, we added different levels OptiCell to the feed of caged and free-range Bian chickens, aiming to research the effects of adding OptiCell and different feeding modes on the microbial diversity and gut health of chickens, and to determine an appropriate amount of OptiCell especially for free-range Bian chickens when plant fibers are lacking in the wild.

2. Materials and Methods

2.1. Animals and Experimental Design

A total of 108 1-day-old Bian chicken roosters were chosen for this experiment (animal study approved the Shanxi Agricultural University Animal Experiment Ethics Committee with approval number SXAU-EAW-2017-002Chi.001), and they were reared in an animal breeding house of Shanxi Agricultural University. They were randomly divided into three groups, each group with six cages and six chickens per cage. OptiCell was supported by the Beijing e-feed & e-vet cooperation. The nutritional matrices of OptiCell are as follows. Nutrients (moisture 8%; crude protein 0.9%; crude fiber 59%; crude ash 1.0%; crude fat 0.8%), fiber fractions (total dietary fiber(TDF) 88%; insoluble TDF 86.7%; soluble TDF 1.3%; NDF 78%; ADF 64%; lignin 25%—30%) and minerals and trace elements (Na 0.03 g/kg; K 0.5 g/kg; Mg 0.2 g/kg; Ca 0.9 g/kg; P 0.1 g/kg; Cu 1.2 mg/kg; Fe 90 mg/kg; Zn 12 mg/kg; Mn 89 mg/kg). Group 1 and Group 2 were given to 1% and 2% OptiCell on the basis of full valence particles, respectively. In contrast, the control group was not given OptiCell.

At the beginning of the ninth week, the chickens were regrouped. Some of chickens were transferred into three-step cages to continue being caged with the same group as before. These chickens were divided into three groups with six reduplicates per group; one cage was one reduplicate with three chickens per cage. Some of the chickens were free-range from September to November. A grassland was divided into three equal areas with net and wood stumps, and three chicken nests were built before the free-range Bian roosters (FBR) came in. Each area was a group, and each group contained 12 chickens. The plant fiber gradually reduced in the wild environment during this period.

Both of the feeding modes involved three kinds of feed, which contained the same energy and protein but different levels of crude fiber due to added OptiCell (0%, 2%, 4%). Group 1 added 2% OptiCell, and was called the OptiCell-low (OL) group, and Group 2 added 4% OptiCell and was called the OptiCell-high (OH) group. The control group was the OptiCell-free (OF) group. This experiment was completed at the end of 20 weeks. Samples were collected to measure the microbial diversity, the thickness of the cecum mucus layer, and the content of SCFAs and GLP-1.

2.2 Management

The chickens were caged in brood cages for the first 1—8 weeks and given free access to water and feed. The management of the indoor temperature, light, and humidity was conducted according to the breeding manual of chickens. From 9—20 weeks, the caged chickens were fed twice a day, morning and night, with free water and food access. The chicken manure was cleaned in a timely manner. We mixed different amounts of OptiCell (Beijing), corn, soybean meal, bran, premix and stone powder (Taigu, Shanxi) to make three kinds of feed with equal energy and protein, but different amounts of fiber (Table 1). The free-range chickens were also fed twice a day. However, they were only fed 60% of the daily feed in the morning to encourage them to eat more plant fiber, and were fed before they returned to the nests in the evening. The free-range area was about 15m² per chicken.

Table 1. Feed formulas of the three kinds of feed used during weeks 9—20.

Components	9—15weeks			16—20weeks		
	OL	OH	OF	OL	OH	OF
Corn%	64.77	64.53	65	66.47	66.7	66.24
Soybean meal%	21.09	22.19	20	21.40	20.3	22.49
Bran%	7.13	4.27	10	2.94	0.073	5.8
OptiCell%	2	4	0	2	4	0
Premix%	5	5	5	5	5	5
Energy MJ/kg	11.50	11.50	11.50	11.47	11.47	11.47
Total Protein %	15.57	15.57	15.57	15.14	15.14	15.14

Total crude fiber%	4.28	5.26	3.30	3.94	5.98	2.95
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2.3. Sampling

We chose six chickens per group at 20 weeks old to collect blood samples from the wing vein after the chickens were fasted for 12 h. Then, the blood tube vessels were bathed in water at 37 °C for 1 h, followed by 3000 × g for 12 min. Following this, the upper serum was absorbed with a pipette into 0.5 mL EP tubes, before being immediately preserved at −20 °C until further GLP-1 analysis.

Following this, the chickens were humanely slaughtered and the length and weights of the intestines were measured. We quickly sampled two pieces from the middle of the right cecum and put them into a carnoy’s fixative solution (the ratio of dry methanol:chloroform:glacial acetic acid was 60:30:10) for 5 h, then changed the samples into fresh carnoy’s solution for 3 h, before washing them in anhydrous methanol for 2 h. Finally, they were changed into new anhydrous methanol and stored at 4 °C until the thickness of the mucus layer was measured [31]. The right cecum contents were collected into two cryogenic tubes, and they were put into a liquid nitrogen tank; then preserved at −80 °C in a fridge until the determination of SCFAs. As above, the left cecum contents were collected to perform the 16S r RNA gene sequence of gut microbiota.

2.4. Determination

2.4.1. 16S r RNA Gene Sequence

The 16S r RNA gene of gut microbiota was sequenced using High-Throughput Sequencing Technology. First, microbial DNA was extracted using the HiPure Stool DNA Kits (Magen, Guangzhou, China). The V3-V4 regions of the 16S ribosomal RNA gene were amplified by PCR using primers 341F 5’-CCTACGGGNGGCWGCAG and 806R 3’-GGACTACHVGGGTATCTAAT. The first PCR reactions were performed in triplicate with 50 μL of mixture containing 5 μL of 10 × KOD Buffer, 5 μL of 2 mM dNTPs, 1.5 μL of each primer (10 μM), 1 μL of KOD Polymerase, and 100 ng of template DNA. The amplification procedure was as follows: 94 °C for 2 min, followed by 30 cycles at 98 °C for 10 s, 62–68 °C for 30 s, and 68 °C for 30 s, and a final extension at 68 °C for 5 min. The second round of amplification was 50 μL mixture containing 5 μL 10 × KOD Buffer, 5 μL 2 mM dNTPs, 3 μL 25 mM MgSO4, 1 μL joint primer (10 μM), 1 μL KOD Polymerase and 100 ng Template DNA, up to 50 μL with ultra-pure water. The amplification procedure was 94 °C 2 min, 98 °C 10 s, 65 °C 30 s, 68 °C 30 s, for 12 cycles, 68 °C for 5 min.

The second step was quality control and reads assembly. Reads filtering, reads assembly, raw tag filtering, chimera checking and removal were successively conducted. Then, the effective tags were clustered into operational taxonomic units (OTUs) of ≥ 97% similarity using the UPARSE pipeline. The tag sequence with the highest abundance was selected as the representative sequence within each cluster. Between groups Venn analysis was performed in R project to identify unique and common OTUs. Then, the taxonomy classification, and α-diversity and β-diversity analysis and functional prediction of these OTUs were successively conducted. The α-diversity reflects the microbial diversity within a single sample, including ACE, chao1, Shannon, Simpson, and so on. The values of ACE and chao1 reflect the community richness. Shannon and Simpson reflect the community richness and community diversity. In contrast, the β-diversity is used to compare the microbial diversity between different samples .

2.4.2. The Concentration of SCFAs

The SCFAs were measured with High Performance Gas Chromatography (HPGC). First, we prepared the deproteinized solution containing internal standard crotonic acid. We accurately weighed metaphosphoric acid 25 g and crotonic acid 0.6464 g, and put them into a 100 mL volumetric flask. Then, we prepared 100 mL mixed standard stock solution (Table 2). The volatile fatty acid standard solution was prepared as follows: 0.2 mL of deproteinized metaphosphate

solution containing crotonic acid was added to three 1.5 mL centrifuge tubes, and 1 mL mixed standard stock solution was added to this.

Table 2. The composition and concentrations of the standard stored liquid.

	acetate	propionate	butyrate	isobutyrate	isovalerate	valerate
supplemented dose μL	330	400	160	30	40	50
final concentration g/L	3.46	3.97	1.53	0.29	0.38	0.47
mol concentration mol/L	57.65	53.63	17.45	3.29	3.67	4.61

Sample preparation: 1 g contents of the cecum was added to nine times the weight of ultra-pure water, homogenated, centrifuged at 10,000 rpm for 10 min, and the supernatant was removed. Then, 1 mL of supernatant sample was placed into a 1.5 mL EP tube, and 0.2 mL mixed solution of crotonic metaphosphate was added, and reacted for 3 h. Centrifugation at 12,000 r for 5 min was undertaken. The supernatant was injected into the chromatograph instantaneously with a 10 μL microinjector, and the injection volume was 1.0 μL.

Then, the conditions were set as follows. The injection temperature was set at 220 °C. The split was 5 and the split ratio was 6. The constant current was 0.8 mL/min. The initial temperature was set at 70 °C and the detector temperature was 220 °C. Tail blowing was 40 ml/min, hydrogen was 35 mL/min, and air was 350 mL/min.

Then, performed the following calculation: the concentration of a certain acid (m mol/L) = (peak area of certain acid of sample × area of standard peak of crotonic acid × standard concentration of certain acid) ÷ (peak area of crotonic acid in sample × area of certain acid in standard sample).

2.4.3. The Thickness of the Mucus Layer

Post carnoy’s fixation, the methanol-stored right cecum samples were dealt with as follows. Washing, dehydration, transparency, embedding, slicing and dewaxing to water were undertaken. Then, sections were dyed with a PA-ABS kit according to the instructions. The thickness of the mucus layer was measured using IP WIN60 software.

2.4.4. GLP-1

We assayed the GLP-1 level in the serum using a Chicken GLP-1 kit. The assay procedures are as follows. First, we prepared the original density standard according to the table provided by the instructions. Then, we began to add samples as follows. (1) Blank wells were set separately from testing sample wells. (2) A total of 50μL of standard was added to microelisa stripplate, and a sample dilution of 40μL was added to the testing sample well, then 10μL of testing sample was added. (3) After closing the plate with a closure plate membrane, it was incubated for 30 min at 37 °C. (4) A 30-fold (or 20-fold) washed solution of configured liquid was diluted 30-fold (or 20-fold) with distilled water and reserved. (5) The closure plate membrane was uncovered, the liquid discarded, and it was dried by swinging before adding washing buffer to every well. They were kept still for 30 s then drained, and this was repeated five times before drying by patting. (6) HRP-Conjugate reagent 50 μL was added to each well, except the blank well. (7) Step (3) was repeated. (8) Step (5) was repeated. (9) Chromogen solution A (50 μl) and shromogen solution B were added to each well, and they were protected from light for preservation for 10 min at 37 °C. (10) Stop Solution (50 μL) was added to each well to stop the reaction, (the reaction was stopped when the blue color changed to yellow). (11) Taking the blank well as zero, the absorbance was read at 450 nm within 15 min after adding the Stop Solution. (12) Taking the standard density as the horizontal and, the OD value for the vertical, the standard curve was drawn on graph paper.

Finding out the corresponding density according to the sample’s OD values, multiplied by the dilution multiple. The unit was pmol/L.

2.5. Statistical Analysis

Statistical analyses were performed using a one-way analysis of variance (ANOVA) with Statistical Product and Service Solutions (SPSS) 22.0 (IBM). The mean ± standard error (SE) was calculated for all data. In terms of microbia, the biomarker features in each group were screened by the linear discriminant analysis effect size (LEfSe) software.

The α-diversity indexes, such as ACE, Chao1, Simpson, and Shannon, were calculated in QIIME. The comparison between groups was calculated by Welch's t-test and Wilcoxon rank test in R project, and the comparison among groups was computed by Tukey’s HSD test and the Kruskal-Wallis H test in R project. The β-diversity analyses of Welch’s t-test, the Wilcoxon rank test, Tukey’s HSD test, and the Kruskal—Wallis H test were calculated using R project. The functional prediction of the OTUs was inferred using FUNGuild.

3. Results

3.1. Gut Microbiota of CBRT

For simplicity, “caged Bian roasts-twenty weeks” was named CBRT for short, and “free-range Bian roasts-twenty weeks” was named FBRT. Both CBRT and FBRT contained three groups, namely the OptiCell-low (OL) group, OptiCell-high (OH) group and OptiCell-free (OF) group. In short, CBRT contained the CBRT-OL, CBRT-OH and CBRT-OF groups. FBRT contained the FBRT-OL, FBRT-OH and FBRT-OF groups.

3.1.1. OTUs of CBRT

The OTUs of CBRT-OL (blue) was less than those of CBRT-OH (green) and CBRT-OF (purple). This means the microbiota of CBRT-OL was the lowest (Figure 1). The three groups shared the majority of OTUs.

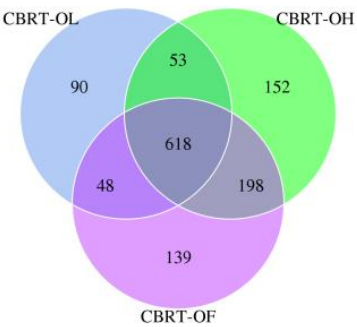


Figure 1. The Venn diagram of OTUs of CBRT.

3.1.2. α-diversity and β-diversity of CBRT

The α-diversity indexes of CBRT-OL were all extremely significantly lower than the other two groups ($p < 0.01$, Table 3), and this was consistent with the Venn diagram. However, the β-diversity was not significantly different among the CBRT groups ($p > 0.05$, Table 3).

Table 3. The comparison of the α-diversity and β-diversity of CBRT.

	CBRT-OL	CBRT-OH	CBRT-OF
α diversity			
ACE	1371.34 ± 97.82 ^B	1810.22 ± 109.35 ^A	1631.94 ± 55.75 ^A
chao1	1375.74 ± 107.08 ^B	1873.99 ± 100.77 ^A	1721.33 ± 95.58 ^A
Shannon	5.86 ± 0.27 ^B	6.55 ± 0.067 ^A	6.57 ± 0.13 ^A

Simpson	0.92 ± 0.021^B	0.97 ± 0.00094^A	0.97 ± 0.0016^A
β diversity	0.16 ± 0.032	0.17 ± 0.039	0.17 ± 0.033

Different superscript letters A,B represent an extremely significant difference in the same line.

3.1.3. The LEfSe of Significant Different Microbiota Among Groups of CBRT

LEfSe is used for finding the OTU of the microbiota biomarkers between groups through Linear Discriminant Analysis (LDA). A value of LDA of certain microbes of >2 represents that the difference was significant between groups (Figure 2).

We observed that the number of dominant bacteria of each group was almost equal(Figure 2).

Because OptiCell is a type of fiber and it can be fermented to produce butyrate, we focused on the significantly different fiber-degradation bacteria, butyrate-producing bacteria, and beneficial bacteria between groups. At the genus level, the relative abundance of *Bacteroides* (41.83%) in CBRT-OL was higher than in CBRT-OH (26.21%) and CBRT-OF (29.59%), respectively, however, *Rikenellaceae_RC9_gut_group* (6.06%) was lower than in CBRT-OH (13.58%) and CBRT-OF (14.17%), respectively (Figure 2a, 2b). At the species level, the relative abundance of *B. sp_SB5* (25.12%) in CBRT-OL was higher than in CBRT-OH (6.26%) and CBRT-OF (5.99%), respectively, however, *B. gallinaceum* (0.19%) was lower than in CBRT-OH (3.85%) and CBRT-OF (7.95%) (Figure 2b, 2c). Compared with CBRT-OF, CBRT-OL and CBRT-OH both had dominant butyrate producers *Faecalibacterium* (Figure 2b 2c) and beneficial bacteria *Akkermansia* (Figure 2b, 2c, 3a, 3b) and CBRT-OH had more low abundance butyrate producers such as *Faecalibacterium*, *Coprococcus_1* and *Oscillospira* (Figure 2c). Notably, the model species *Akkermansia muciniphila* of *Akkermansia* is also a mucus-eroding microbiota.

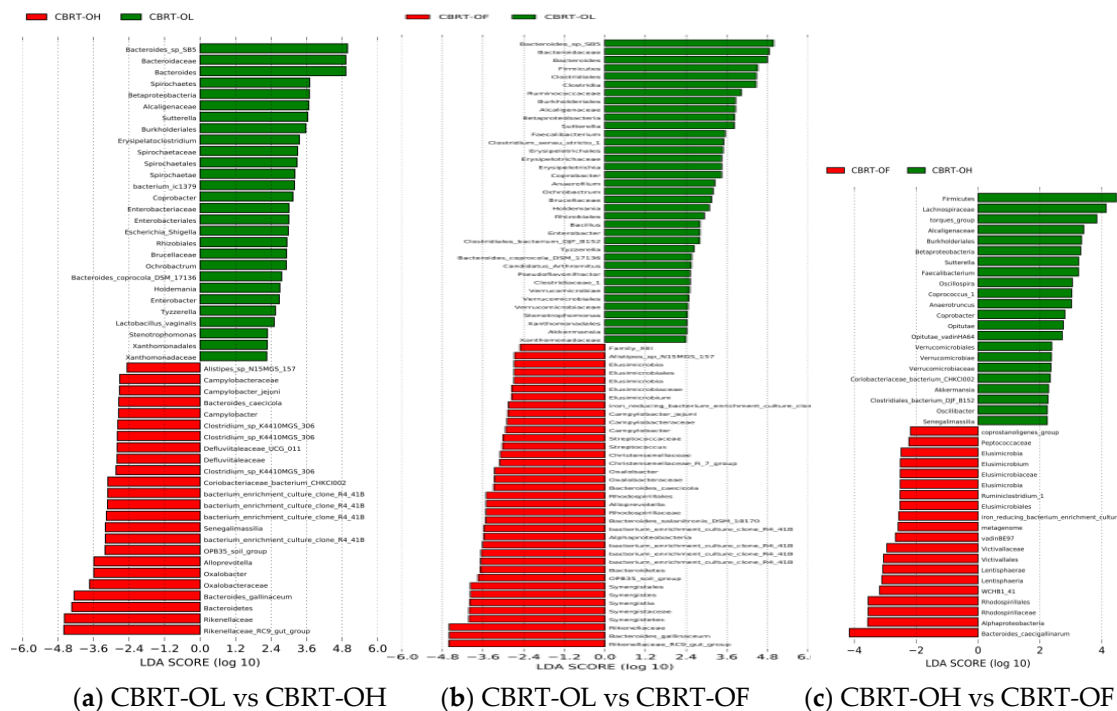
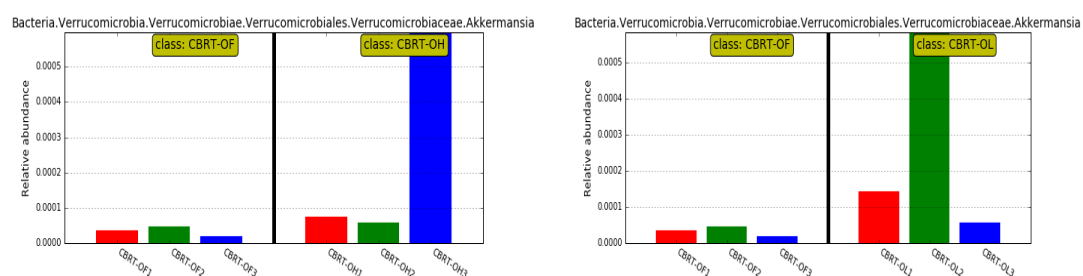


Figure 2. LDA among the groups of CBHT.



(a) CBRT-OF vs CBRT-OH (b) CBRT-OF vs CBRT-OF

Figure 3. Abundance histogram of Akkermansia between the groups of CBRT.

3.2. Gut Microbiota of FBRT

3.2.1. OTUs of FBRT

The OTUs of FBRT-OL and FBRT-OH were both more than FBRT-OF. This means adding OptiCell could increase the gut microbial diversity of free-range chickens. The OTUs of FBRT-OL were the highest (Figure 4), and this means the microbiota of the 2% level OptiCell group was the highest.

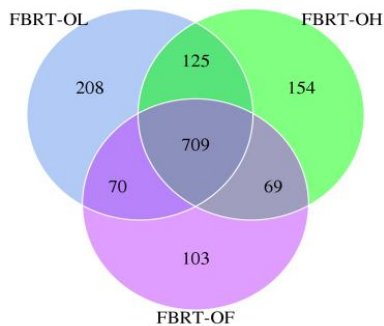


Figure 4. The Venn diagram of OTUs of FBRT.

3.2.2. α-diversity and β-diversity of FBRT

The α-diversity of FBRT-OL was significantly higher than FBRT-OF, except for the chao1 index ($p < 0.05$, Table 4). The β-diversity of FBRT-OH was also significantly higher than FBRT-OF ($p < 0.01$, Table 4). This suggested that adding OptiCell could increase the microbial diversity of FBRT.

Table 4. The comparison of α-diversity and β-diversity of FBRT.

	FBRT-OL	FBRT-OH	FBRT-OF
α-diversity			
ACE	1773.31 ± 43.86 ^a	1772.66 ± 58.93 ^a	1524.77 ± 6.28 ^b
chao1	1818.33 ± 51.32	1799.93 ± 76.45	1616.26 ± 30.42
Observed species	1251.33 ± 33.20 ^a	1213.67 ± 49.39 ^b	1108.67 ± 4.37 ^b
Shannon	7.19 ± 0.056 ^a	6.87 ± 0.12 ^{ab}	6.66 ± 0.052 ^b
Simpson	0.98 ± 0.00041	0.97 ± 0.0035	0.97 ± 0.0037
β-diversity	0.15 ± 0.025 ^{AB}	0.23 ± 0.061 ^A	0.097 ± 0.0043 ^B

Different superscript letters (A,B) and (a,b) separately represent an extremely significant and a significant difference in the same line.

3.2.3. The LEfSe of Significantly Different Microbiota Among FBRT

Compared with FBHT-OF, FBRT-OL and FBRT-OH both had more of the low abundance butyrate producers *Roseburia* and *Faecalibacterium*. *Roseburia* can also effectively degrade dietary fiber (Figure 5a, 5b). The number of butyrate producers in FBRT-OH was more than in FBRT-OF, including *Faecalibacterium*, *Anaerostipes* and *Subdoligranulum* (Figure 5c). However, at the genus level, the relative abundance of *Bacteroides* (33.54%) in FBRT-OF was higher than in FBRT-OL (19.55%) and FBRT-OH (20.07%), respectively (Figure 5b, 5c). At the species level, the relative abundance of *B. caecigallinarum* (11.15%) in FBRT-OF was higher than in FBRT-OL (2.69%) and FBRT-OH (2.71%), respectively (Figure 5b, 5c).

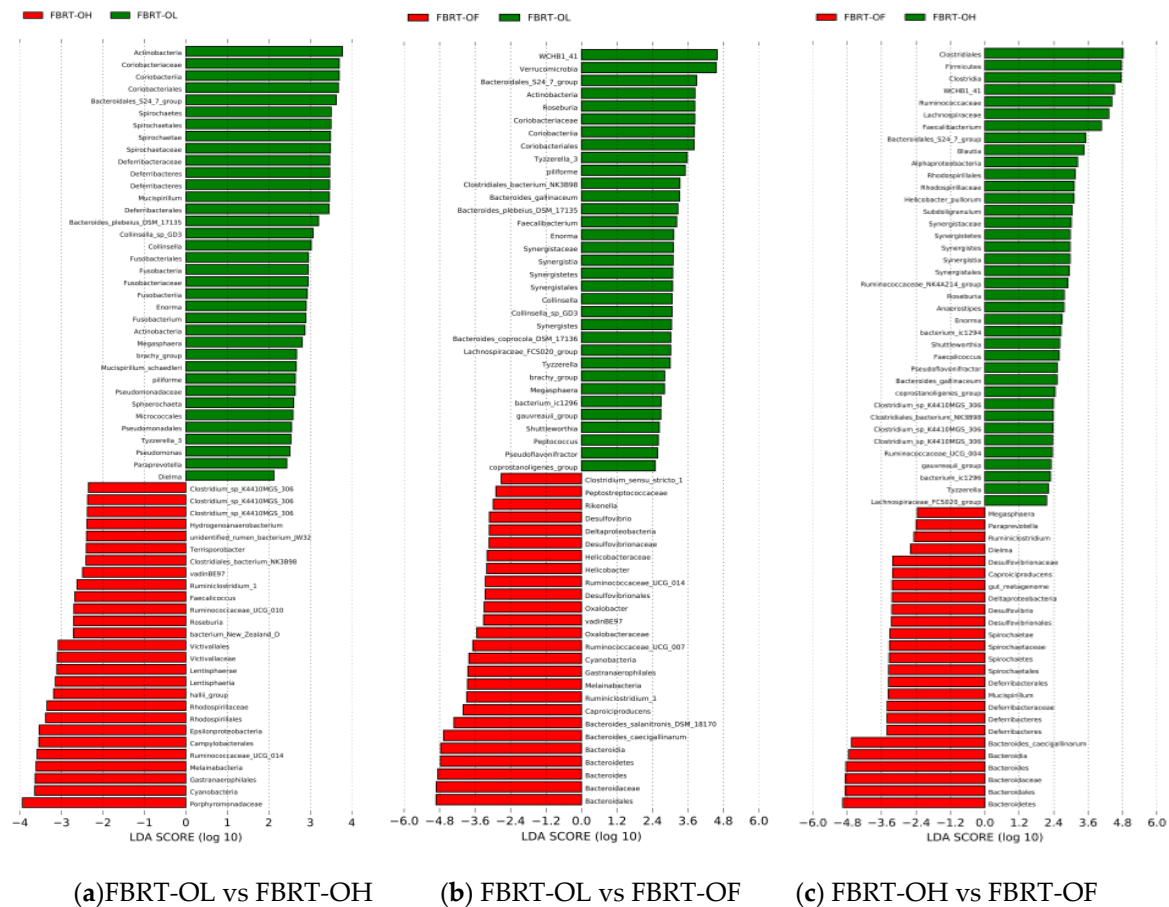


Figure 5. LDA among the groups of FBHT.

3.2.4. Comparison of Dominant Gut Microbiota Among FBRT

FBRT-OL had more dominant bacteria, including the butyrate producers *Megasphaera* and fiber-degrader *Paraprevella*. FBRT-OH also had *Roseburia* which is good at degrading fiber and producing butyrate. (Figure 6).

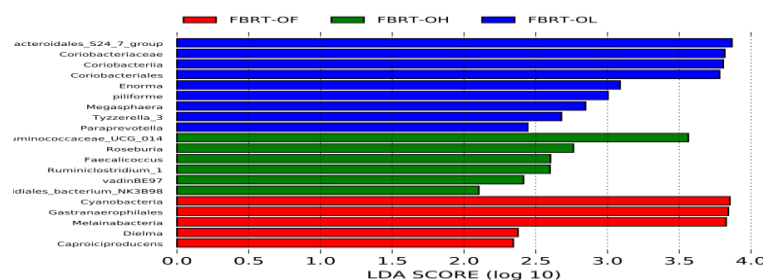
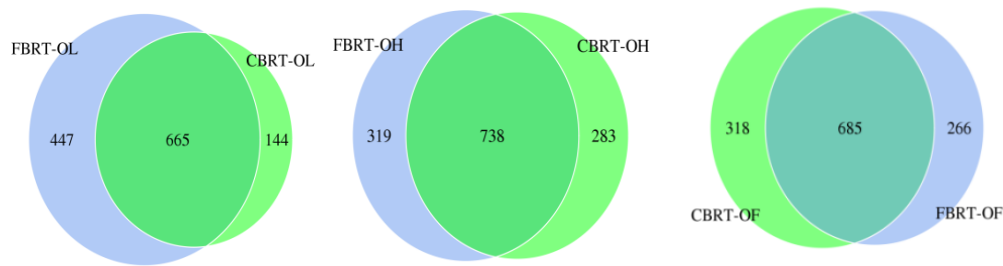


Figure 6. Abundance histograms of significantly different gut microbiota among FBRT.

3.3. The Comparison of Gut microbiota between CBRT and FBRT

3.3.1. The Comparison of OTUs between CBRT and FBRT

The OTUs of the three groups of FBRT were more than in CBRT, except the OF group (Figure 7). This indicated that only groups which had added OptiCell increased their gut microbiota compared with CBRT, and also indicated that it is necessary to add dietary fiber to the feed of free-range chickens, especially when plant fibers are lacking. Notably, the unique OTUs of FBRT-OL were higher than in CBRT-OL (Figure 7a).



(a) FBRT-OL vs CBRT-OL (b) FBRT-OH vs CBRT-OH (c) CBRT-OF vs FBRT-OF

Figure 7. The Venn diagrams of OTUs of CBRT and FBRT

3.3.2. Comparison of α -diversity and β -diversity between CBRT and FBRT

The α -diversity indexes of FBRT-OL were all extremely significantly higher than CBRT-OL ($p < 0.01$, Table 5). However, the β -diversity showed no difference among the groups of CBRT ($p > 0.05$).

Table 5. The comparison of α -diversity and β -diversity between CBRT and FBRT.

	FBRT-OL	CBRT-OL	FBRT-OH	CBRT-OF	FBRT-OF	CBRT-OF
α -diversity						
ACE	1773.31 $\pm 43.86^A$	1371.34 $\pm 97.82^B$	1772.66 ± 58.93	1810.22 ± 109.35	1524.77 ± 6.28	1631.94 ± 55.75
chao1	1818.33 $\pm 51.32^A$	1375.74 $\pm 107.08^B$	1799.93 ± 76.45	1873.99 ± 100.77	1616.26 ± 30.42	1721.33 ± 95.58
Shannon	7.19 $\pm 0.056^A$	5.86 $\pm 0.27^B$	6.87 ± 0.12	6.55 ± 0.067	6.66 ± 0.052	6.57 ± 0.13
Simpson	0.98 $\pm 0.00041^A$	0.92 $\pm 0.021^B$	0.97 ± 0.0035	0.97 ± 0.00094	0.97 ± 0.0037	0.97 ± 0.0016
β -diversity	0.15 ± 0.025	0.16 ± 0.032	0.23 ± 0.061	0.17 ± 0.039	0.097 ± 0.0043	0.17 ± 0.033

Different superscript letters A, B represent an extremely significant difference in the same line.

3.3.3. The LEfSe of Significant Different Microbiota among CBRT and FBRT

The number of dominant bacteria of FBRT-OL and FBRT-OH were more than in CBHT-OL and FBRT-OH, respectively (Figure 8a, 8b). The relative abundance of *Bacteroides* in CBRT-OL (41.83%) was higher than in FBRT-OL (19.55%), however, *Rikenellaceae_RC9_gut_group* (6.06%) was lower than in CBRT-OL(13.16%) (Figure 8a). Compared with CBRT-OF, FBRT-OF had more of the dominant bacteria *Akkermansia* (Figure 8c, 9).

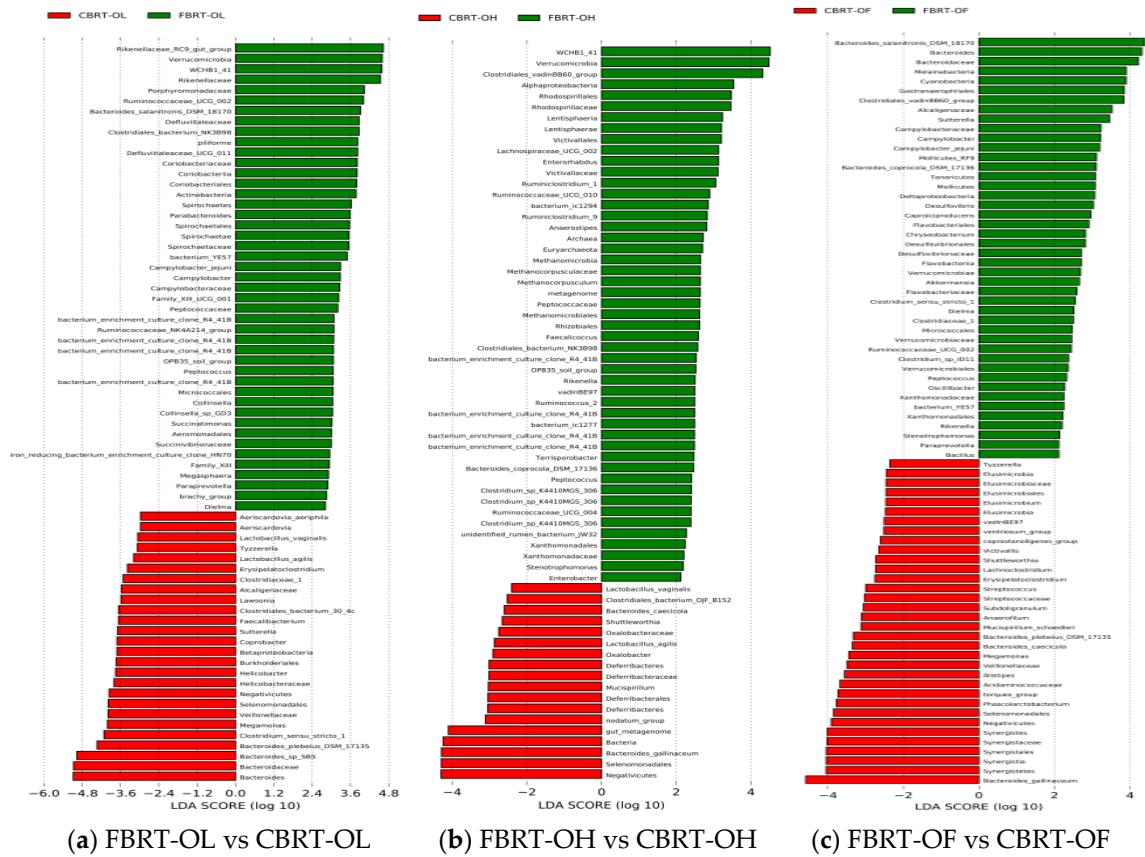


Figure 8. LfSe of microbiota in FBHT and CBHT.

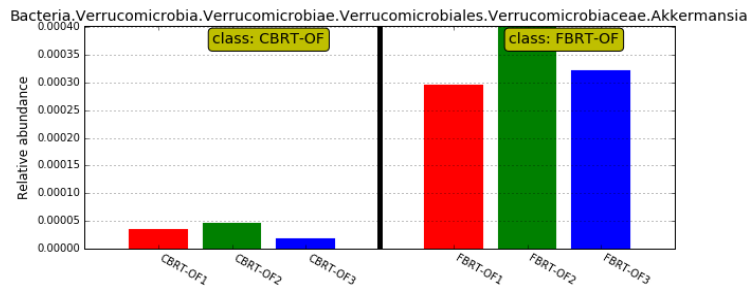


Figure 9. Abundance histogram of *Akkermansia* in CBRT-OF and FBRT-OF.

Because the α -diversity in FBRT-OL and CBRT-OL was significantly different, we plotted the evolutionary branch tree diagram between them according to the LfSe results (Figure 10). Compared with CBRT-OL (red), the dominant bacteria in FBHT-OL was *Actinobacteria* (green).

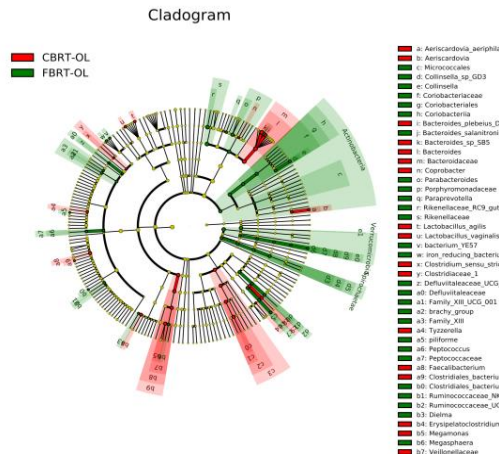


Figure 10. The evolutionary branch tree diagram of significantly different gut microbiota between the CBRT-OL and FBHT-OL groups.

3.4. Functional Prediction of OTUs of CBRT-OL and FBRT-OL

Next, we performed a functional prediction of OTUs in CBRT-OL and FBRT-OL. This showed that the function of gut microbiota of CBRT-OL (Figure 11a) was similar to that of FBRT-OL (Figure 11b). Both were focused on carbohydrate metabolism, amino acid metabolism, metabolism of cofactors and vitamins, energy metabolism, membrane transport, and signal transduction.

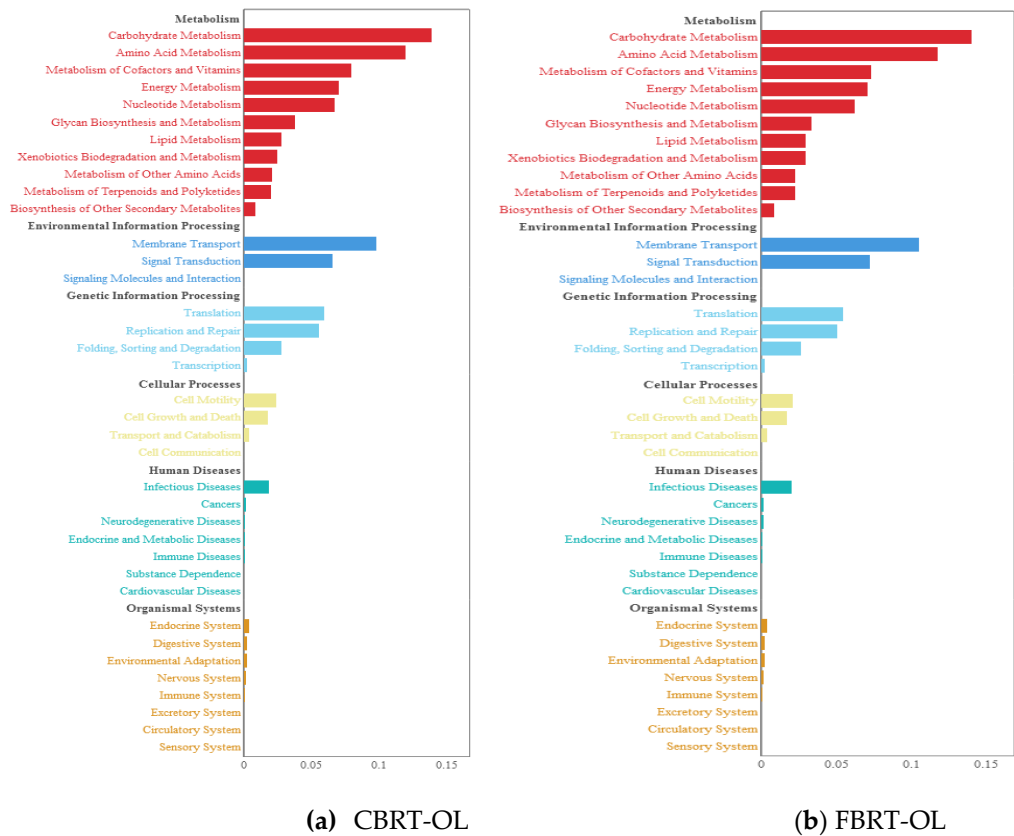


Figure 11. Functional prediction of OTUs of FBRT-OL and CBRT-OL.

3.5. Comparison of the Length of Small Intestine between FBRT and CBRT

The length of small intestine of FBRT was longer than CBRT by 9–17cm, and FBRT-OL was significantly longer than CBRT-OL (Table 6). The length and weight of the cecum of FBRT also was larger than CBRT, except in the case of FBRT-OH. This suggested that free-range rearing benefited the development of the small intestines, while the development of the cecum was limited by high OptiCell.

Table 6. The development of the small intestine and cecum of FBRT and CBRT.

		OL	OH	OF
FBRT	small intestines (cm)	125.75 ± 5.24 ^a	120.2 ± 6.69	124.83 ± 4.90
CBRT	small intestines (cm)	108.58 ± 3.91 ^b	111.3 ± 3.05	112.47 ± 6.41
FBRT	cecum (cm)	13.6 ± 0.50	13.1 ± 0.61	14.92 ± 0.81
CBRT	cecum (cm)	13 ± 1.08	13.67 ± 0.48	12.62 ± 0.34
FBRT	cecum (g)	5.24 ± 0.23	4.38 ± 0.23	5.59 ± 0.26 ^a
CBRT	cecum (g)	4.47 ± 0.53	4.96 ± 0.36	4.64 ± 0.27 ^b

Different superscript letters a, b represent a significant difference in the same column.

3.6. The Effects of Adding OptiCell and Different Feeding Modes on SCFAs

3.6.1. The Effect of Adding OptiCell on SCFAs

There was no significant difference either in CBRT or FBHT, except that the isobutyrate of CBHT-OH was lower than CBHT-OF ($p < 0.05$) (Table 7).

Table 7. The concentration of SCFAs of the different groups.

	acetate	propionate	butyrate	isobutyrate	isovalerate	valerate
CBHT-OL	4.28 ± 1.51	1.30 ± 0.44	0.64 ± 0.30	0.12 ± 0.036 ^{ab}	0.13 ± 0.046	0.15 ± 0.047
CBHT-OH	4.09 ± 0.17	1.50 ± 0.22	0.58 ± 0.12	0.086 ± 0.003 ^b	0.09 ± 0.006	0.12 ± 0.011
CBHT-OF	5.20 ± 2.20	1.82 ± 0.31	0.80 ± 0.40	0.23 ± 0.081 ^a	0.32 ± 0.19	0.17 ± 0.055
FBHT-OL	4.50 ± 0.77	1.58 ± 0.41	1.00 ± 0.44	0.18 ± 0.057	0.27 ± 0.075	0.18 ± 0.059
FBHT-OH	4.57 ± 0.28	1.37 ± 0.04	0.63 ± 0.078	0.13 ± 0.014	0.17 ± 0.034	0.14 ± 0.005
FBHT-OF	5.67 ± 0.56	1.97 ± 0.26	0.92 ± 0.21	0.14 ± 0.023	0.22 ± 0.037	0.16 ± 0.017

Different superscript letters a, b represent a significant difference in the same column.

3.6.2. The Effects of Different Feeding Modes on SCFAs

There was no significant difference in others except that the isobutyrate of CBHT-OH was lower than FBHT-OH ($p < 0.05$) (Table 8).

Table 8. The concentration of SCFAs in CBRT and FBRT.

	acetate	propionate	butyrate	isobutyrate	isovalerate	valerate
CBHT-OL	4.28 ± 1.51	1.30 ± 0.44	0.64 ± 0.30	0.12 ± 0.036	0.13 ± 0.046	0.15 ± 0.047
FBHT-OL	4.50 ± 0.77	1.58 ± 0.41	1.00 ± 0.44	0.18 ± 0.057	0.27 ± 0.075	0.18 ± 0.059
CBHT-OH	4.09 ± 0.17	1.50 ± 0.22	0.58 ± 0.12	0.086 ± 0.003 ^b	0.09 ± 0.006	0.12 ± 0.011
FBHT-OH	4.57 ± 0.28	1.37 ± 0.036	0.63 ± 0.078	0.13 ± 0.014 ^a	0.17 ± 0.034	0.14 ± 0.005
CBHT-OF	5.20 ± 2.20	1.82 ± 0.31	0.80 ± 0.40	0.23 ± 0.081	0.32 ± 0.19	0.17 ± 0.055
FBHT-OF	4.36 ± 0.25	1.46 ± 0.068	0.51 ± 0.02	0.15 ± 0.043	0.18 ± 0.030	0.13 ± 0.016

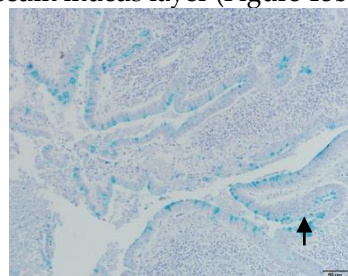
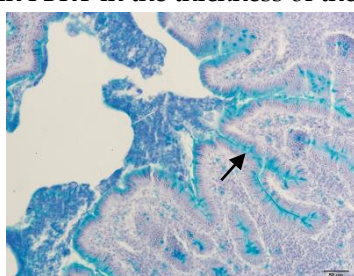
Different superscript letters a, b represent a statistically significant difference in the same column.

In addition, we also detected the concentration of SCFAs in the hypothalamus. We found that acetate was the main SCFA. The average value of acetate in the three groups of CBHT was 1.27 ± 0.38 , while in FBHT it was 0.37 ± 0.028 . Though the difference between them was not significant, the concentration in the hypothalamus in CBRT was higher.

3.7. The Thickness of the Cecum Mucus Layer

3.7.1. The Effects of OptiCell on the Thickness of the Cecum Mucus Layer

The blue substance is the mucus layer, indicated by the arrows. The thickness of the cecum mucus layer in CBRT-OL (Figure 12a) was greater than in CBRT-OF (Figure 12b, 13a). This suggested that added OptiCell could increase the thickness of the mucus layer of the cecum. There was no difference in FBRT in the thickness of the cecum mucus layer (Figure 13b).



(a) CBRT-OL (b) CBRT-OF

Figure 12. The thickness of the cecum mucus layer.

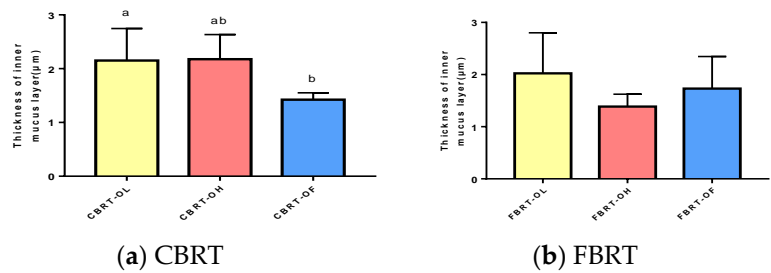


Figure 13. Histograms of the thickness of the cecum mucus layer in the different groups.

3.7.2. The Effects of Different Feeding Modes on the Thickness of the Cecum Mucus Layer

The thickness of the cecum mucus layer in CBRT-OH was greater than in FBRT-OH (Figure 14, 15). This was consistent with the lesser development of the cecum in FBRT-OH.

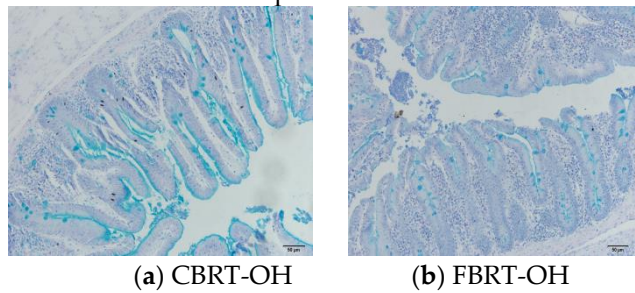


Figure 14. The thickness of the cecum mucus layer.

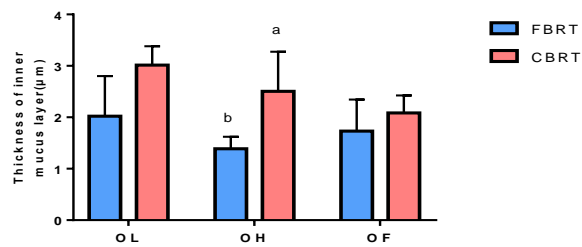


Figure 15. Histograms of the thickness of the cecum mucus layer in CBRT and FBRT.

3.8. GLP-1

3.8.1. The Effect of Adding OptiCell on the GLP-1

There was no significant difference among any of the three groups of CBRT or CBRF (Table 9).

Table 9. The GLP-1 of CBRT and CBRF.

	OL	OH	OF
CBRT	12.46 ± 0.16	11.58 ± 0.26	12.05 ± 0.56
FBRT	8.94 ± 0.14	9.91 ± 0.38	10.20 ± 0.093

3.8.2. The Effects of Different Feeding Modes on the GLP-1

The GLP-1 of CBRT was higher than FBRT ($p < 0.01$) (Table 10). This suggested that caged chickens were more satiated compared with free-range chickens.

Table 10. The comparison of GLP-1 between CBRT and CBRF.

	OL	OH	OF
CBRT	12.46 ± 0.16 ^A	11.58 ± 0.26 ^A	12.05 ± 0.56 ^a
FBRT	8.94 ± 0.14 ^B	9.91 ± 0.38 ^B	10.20 ± 0.093 ^b

Different superscript letters (A, B)and (a, b) represent an extremely significant difference and a significant difference in the same column respectively.

4. Discussion

4.1. The Effects of Adding OptiCell and Different Feeding Modes on Gut Microbial Diversity

Improving the fiber level can increase the gut microbial diversity of the host. In this experiment, adding OptiCell increased the gut microbial diversity of free-range chickens. However, the α -diversity of CBRT-OL was the lowest in the caged groups. This was related to the feed formula. Though the fiber content of CBRT-OF was the lowest, the content of bran was the highest. Bran is a fiber substrate of microbiota, so it increased the microbial abundance. This also suggested that we should not only focus on the fiber content while ignoring the effects of other components in feed formulas on microbial diversity. However, the α -diversity of CBRT-OH was higher than in CBRT-OL, suggested that adding OptiCell could increase gut microbial diversity.

The gut microbial diversity of free-range chickens was higher than that of caged chickens [32]. This was mainly driven by consumption of various plant fibers. The diversity of herbivore bacteria is more abundant [33]. By contrast, a decrease in the diversity of food sources reduced the gut microbial diversity [34]. In this experiment, we also found free-range chickens have more unique gut microbiota than caged chickens, except for the OF group. This indicated that varied gut microbiota depends on the diversity of food, and it also indicated that it is necessary to add the dietary fiber OptiCell to the feed of free-range chickens when plant fibers are lacking.

In addition, we found that *Actinobacteria*, which is the main phylum in soil, was more abundant in the FBRT-OL group of free-range chickens than caged chickens. *Actinobacteria* can produce various natural drugs and bioactive metabolites, which benefits the health of chickens [32]. Chickens usually look for worms in soil and forage gravels for grinding food. In addition, the length of the small intestine of free-range chickens was longer than caged chickens, and this could be attributed to this special habit of eating gravel.

4.2 The Effects of Adding OptiCell and Different Feeding Modes on Akkermansia and the Cecum Mucus Layer

The mucus layer is the first physical barrier, and it can prevent aggression by enteric pathogens. The mucus layer is mainly composed of MUC2 mucin secreted by goblet cells [35]. The mucus layer consists of two layers; the outer mucus layer is loose and allows the microbial colonization, while the inner mucus layer is dense and contains little bacteria, as a defense against enteric pathogens.

Akkermansia muciniphila is the model bacteria of *Akkermansia*. It is a typical mucus-eroding microbiota and also a beneficial bacteria. It has been found that increasing dietary fiber can increase the thickness of the mucus layer and the abundance of *A. muciniphila* [36], which protects the gut from colitis disease [37]. However, other research has shown that a lack of dietary fiber leads to an increase of *A. muciniphila* and thinning of the mucus membrane [38]. In addition, butyrate can also stimulate the synthesis of mucin glycoprotein and enhance the protective effect on the mucus membrane. The butyrate producer *Roseburia intensinalis* is specialized in colonization in mucin [39].

In this experiment, the thickness of the cecum mucus layer and the relative abundance of *Akkermansia* increased in both CBRT-OL and CBRT-OH. This indicated that added OptiCell could increase these measures. Because mucin is a potential growth substrate of *A. muciniphila* [40], the increase of the thickness of the mucus layer promoted *Akkermansia* reproduction, and these factors came together to improve the gut health of the host. Interestingly, *A. muciniphila* can also stimulate the production of mucus and the expansion of goblet cells which secret mucus [41], and increase the thickness of mucus layer in turn. In addition, the thickness of the cecum mucus layer in CBRT-OH

was thicker than in FBRT-OH, suggesting it may not be good for mucosal health of free-range chickens if too much OptiCell is added.

4. 3. The Effects of Adding OptiCell and Different Feeding Modes on SCFAs

Dietary fiber can be fermented by microbiota to produce short chain fatty acids (SCFAs), including acetate, propionate, butyrate, isobutyrate, isovalerate, valerate, and so on. Acetate, propionate and butyrate account for 90%~95% of SCFAs. OptiCell fermentation can produce a lot of lactate [42] and butyrate. Butyrate-producing genera include *Ruminococcus*, *Faecalibacterium*, *Eubacterium*, *Roseburia*, *Coproccoccus*, *Anaerostipes*, and more.

In this experiment, the effects of adding OptiCell and different feeding modes on SCFAs were both small. In addition, compared with the group OF, the dominant bacteria in OL and OH included *Faecalibacterium* or *Roseburia*. However, there was no significant difference in the concentration of butyrate among groups. This may be because the relative abundance of these butyrate producers was low, or butyrate was absorbed more in the groups OL and OH.

4. 4. The Effects of Adding OptiCell and Different Feeding Modes on GLP-1

In addition to supplying energy, SCFAs can also stimulate the release of glucagon like peptide-1 (GLP-1) by activating GPR41 (renamed FFAR3) and GPR43 (FFAR2). GLP-1 is an incretin hormone which is secreted by intestinal endocrine L cells, making the host feel satiety and reducing intake. The GLP-1 of the free-range chickens was lower in this experiment, indicating that they were hungrier than the caged chickens. Given the SCFAs were not significantly different between CBRT and FBRT, we speculate that this was mainly caused by the energy of the diets rather than SCFAs. The free-range chickens lacked energy, so they were more hungrier. This showed that energy is also an important factor affecting GLP-1.

In addition, some studies have also proven that acetate could, through a central mechanism independent of GLP-1, cause anorexia [43] or increased appetite [44] via the hypothalamus. Therefore, we detected the content of acetate in the hypothalamus, to test whether this difference in appetite was driven by this mechanism. However, we found that the value of acetate was no significantly different between CBRT and FBRT, though the value of acetate in CBRT was higher than FBRT. Therefore, energy is the main factor which contributed to this difference in appetite.

5. Conclusions

Adding OptiCell can increase the thickness of the cecum mucus layer and benefits the gut health of caged chickens and 4% OptiCell is optimum. In addition, adding OptiCell can increase microbial diversity and the abundance of some butyrate-producing bacteria in free-range chickens.

However, neither adding OptiCell nor different feeding modes have much effects on the content of SCFAs. The significant difference of GLP-1 between CBRT and FBRT is mainly driven by energy rather than SCFAs or acetate, which was measured in the hypothalamus in this experiment.

The free-range mode promotes the development of the small intestine. In addition, the effects of different feeding modes, especially fiber source diversity on gut microbial diversity were greater than that obtained using OptiCell. Free-range chickens also need to be given an appropriate amount of dietary fiber to improve microbial diversity and gut health when there is a lack of plant fiber in the wild, and 2% OptiCell is optimum for them according to this experiment.

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

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