Comparative Study of Endogenous Ileal Amino Acid Flow and Digestive Physiology to Nitrogen-Free Diets with Differing Ratios of Dextrose to Corn Starch in Broiler Chickens

Huajin Zhou
China Agricultural University
Wei Wu
China Agricultural University
Tahir Mahmood
Adisseo Animal Nutrition, DMCC, Dubai, United Arab Emirates
Yanhong Chen
China Agricultural University
Yanwei Xu
China Agricultural University
Youli Wang
China Agricultural University
Jianmin Yuan (yuanjm@cau.edu.cn)
China Agricultural University

Research Article

Keywords: Broiler, Ileal endogenous amino acids, Nitrogen-free diets

Posted Date: July 9th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-677867/v1

Abstract

Determination of ileal endogenous amino acids (IEAAs) is necessary for the calculation of standardized ileal amino acid digestibility. This experiment was conducted to compare the response of amino acids composition of IEAAs of broilers, and digestive physiology fed the nitrogen-free diet (NFD) formulated with different ratios of dextrose to corn starch (D/CS). 28d-old broiler chickens (n = 210) with similar body weight were allocated to 5 treatment groups, including a control group (CT, basal diet, normal level of protein) and four NFD groups for a 3-days trial, designated as A (D/CS = 1.00), B (D/CS = 0.60), C (D/CS = 0.33), and D (D/CS = 0.14). The results showed that NFD significantly reduced serum IGF-1, albumin and uric acid levels when compared with the control ($P < 0.05$). A higher ratio of D/CS (1.00 and 0.60) increased Asp, Thr, Ser, Glu, Gly, Ala, Val, Ile, Leu, His, Tyr, Arg, and Pro contents of IEAAs when compared with the ratio of 0.33 and 0.14 ($P < 0.05$). Moreover, ileal DM digestibility and digestive enzyme increased with an increasing ratio of dextrose to corn starch ($P < 0.001$). The number of ileal goblet cells and the gene expression of Mucin 2 were higher in group A (D/CS=1.00) than in group C (D/CS = 0.33) and the control ($P < 0.05$). It was further observed that NFD indeed reshaped the gut microbiota, characterized by lower Bacteroidetes, a significantly increased proportion of Proteobacteria, and decreased microbial diversity ($P < 0.05$).

Our results indicate that the chicken fed NFD were accompanied by huge digestive physiological alterations, presenting with malnutrition and accumulation of Proteobacteria in the gut. Different proportions of dextrose and starch directly affect the basal IEAAs of broiler chickens. A higher proportion of dextrose (D/CS = 1 and 0.6) in NFD increase IEAAs by promoting secretion of digestive enzyme and mucin. But the excessive proportion of starch is unsuitable for the chicken to digest NFD (D/CS = 0.14). Therefore, we suggest the ratio of dextrose to corn starch in NFD at 0.33 might be more appropriate to detect IEAAs of broiler chickens.

Introduction

Accurate determination of amino acid digestibility of raw materials is arguably the critical foundation of feed formulation in poultry production. Such information helps to improve protein utilization and minimize N losses. Dietary formulas based on apparent ileal amino acid digestibility (AID) has been widely used. Nevertheless, AID underestimates the actual digestibility of amino acids in broilers by neglecting the endogenous ileum amino acids (IEAAs) losses. This is particularly true for low CP ingredients, such as cereal grains [1]. The IEAAs are considered as an inevitable loss, consisting of salivary and gastric secretions, pancreatic and bile secretions, small intestinal secretions, mucus, sloughed epithelial cells, and microbial protein [2, 3]. The formula based on standardized ileal digestibility (SID) can correct the AID by accounting IEAAs losses induced by the protein-free ingredients. Undoubtedly, SID can more accurately reflect the digestibility of feed protein, thereby measuring IEAAs losses to determine the SID of AAs is necessary for accurate diet formulation.

In monogastric animals fed on nitrogen-free diets (NFD), the IEAAs losses have traditionally been determined by measuring AA excretion in the ileal digesta [4]. Corn starch and dextrose or sucrose are the main components of the NFD (approximately 80% of NFD) [5–8]. Interestingly, corn starch to dextrose or sucrose ratio varies (0.31 to 9.52) in literature [5–8]. This clearly indicates that there is no unified standard for the proportion of starch and dextrose in the NFD formula. More importantly, the putative effects of the different ratios of corn starch to dextrose in NFD on IEAAs are hard to ignore. Some reports in broiler chickens identified that total IEAAs were significantly higher when dextrose was used as a sole source of energy in NFD compared with corn starch (17544 vs. 12779 mg/kg of dry matter intake) [9]. Similar findings were reported in broilers study, wherein the IEAAs loss in NFD containing only glucose was significantly higher than that in NFD containing only corn starch (11080 vs. 6038 mg/kg of dry matter intake) [10].These studies clearly indicated the influence of the varying ratios of corn starch and dextrose on IEAAs losses, thereby affecting the accuracy of the SID. Arguably, these studies did not present the possible explanation for the difference in IEAAs loss brought in by the ratio of starch to dextrose.

Thus, we hypothesized that the composition and flow of IEAAs losses resulting from an ideal EIAAs flow should be evaluated in the normal digestive physiological state, which is also the reason why the chickens fed with basal diet was served as a control group in this study. Here, the main components of endogenous flow were estimated, for instance, mucus, digestive

enzymes, intestinal microbial composition as well as intestinal health status. This can help to develop a better formula of NFD by comparing the main components of IEAAs loss and intestinal health status with the chickens under normal condition.

Therefore, the main objective of the present study was to determine the influence of varying ratios of dextrose and corn starch in the NFD on endogenous ileal amino acid flow and digestive physiology in broiler chickens.

Results

Serum Metabolites

It was found that the NFD diets significantly decreased serum concentrations of albumin and uric acid of broiler chickens when compare with CT group (Table 4, $P < 0.001$), and the IGF-1 level was slightly but not significantly higher in group C (D/CS = 0.33) than the group D (D/CS = 0.14, $0.05 < P < 0.1$). However, there were no statistically differences in insulin, glucose, glucagon, and TP among groups.

IEAAs losses

In this study, a significant decrease in most IEAAs losses can be observed when the ratio of dextrose to starch in the NFD dropped below 0.6 ($P < 0.05$). A higher ratio of D/CS (1.00 and 0.60) increased the basal endogenous losses of most amino acids including Asp, Thr, Ser, Glu, Gly, Ala, Val, Ile, Leu, His, Tyr, Arg, and Pro than that of the lower ratio of D/CS (0.33 and 0.14) (Table 5, $P < 0.05$). The highest basal endogenous loss of Phe was detected at a D/CS ratio of 0.60 and which was significantly higher than that in the D/CS ratio of 0.33 and 0.14 ($P < 0.05$). In addition, the basal endogenous loss of Lys was significantly higher at a D/CS ratio of 1.00 than 0.14 ($P < 0.05$). These results indicated that different dietary carbohydrate composition substantially affects excretion of endogenous amino acids by poultry.

Intestinal morphology

As shown in Table 6, the number of goblet cells of jejunal and ileal villi was larger in the NFD groups when compared to control ($P < 0.05$), but there was no change on the duodenum. In ileum, the increasing trend of villus height in the NFD groups was obvious ($0.05 < P < 0.1$), especially group B had the highest villus, whereas the crypt depth in the NFD groups showed a decreasing trend ($0.05 < P < 0.1$). Thus, the ratio of villus height to crypt depth (V/C) of ileum differed significantly among the groups, it was higher in NFD groups than control group ($P < 0.05$), and the V/C of duodenum had a similar trend of rising in the NFD groups ($0.05 < P < 0.1$).

Digestive enzyme activity

The digestive enzymes constitute an important component of endogenous losses. Thus, the activity of five important digestive enzymes was determined in this study. As shown in Table 7, the α-amylase and lipase levels showed a significant rise at the D/CS ratio of 1.00, compared to 0.14, and the control ($P < 0.05$). However, the chymotrypsin level in D/CS 1.00 and the control was remarkably reduced compared with that from D/CS ratios of 0.60 and 0.14 ($P < 0.05$). As for mucosal disaccharidase, the highest level of sucrase was found in group A (D/CS = 1.00), the lowest level of sucrase was found in D (D/CS = 0.14, $P < 0.05$) and there was no significant difference among group B (D/CS = 0.60), D (D/CS = 0.14) and the control group. In addition, group A (D/CS = 1.00) also had the highest level of maltase, and this difference was significant compared to group D (D/CS = 0.14) and the control ($P < 0.05$). Most notably, there was no significant difference in the activities of five digestive enzymes between NFD and control group when the ratio of D/CS in NFD was 0.33.

The AID of DM

Furthermore, group A (D/CS = 1.00) also showed a significant rise in dry matter digestibility (Fig. 1), when compared to the control ($P < 0.05$). There was no significant difference among groups B (D/CS = 0.60), group C (D/CS = 0.33), and the control group. However, the lowest dry matter digestibility was observed in group D (D/CS = 0.14), which was extremely lower than the

control group ($P < 0.001$), indicating that the starch composition is too high for the chicken to digest when the dextrose to starch in NFD is 0.14.

Gene expression

Intestinal cell types are related to intestinal function and the composition of endogenous amino acids. For example, mucus secreted by goblet cells is an important component of endogenous amino acids loss. Therefore, the intestinal cell marker genes were detected in this study. As shown in Fig.2, we demonstrate that the goblet cell marker gene Mucin2 were significantly higher in D/CS ratio of 1.00 and 0.60 when compared with the control ($P < 0.05$). NFD treatment corresponded to upward trends of mucous glucose transporters, both GLUT2 and SGLT1 levels were significantly higher in NFD groups than the control ($P < 0.05$).

Microflora analysis of digesta in the ileum

According to the results of 16S sequencing based on operational taxonomic units (OTUs) analysis, the microbiota diversity and composition in the ileum of control and NFD treated chickens were differed markedly at different levels, respectively (Fig. 3a, b). The principal coordinates analysis (PCoA) of the weighted UniFrac distances resulted in a significant segregation among groups (Fig.3c), confirming the presence of compositional differences of microbiota in the ileum of NFD treatments and the control. As shown in Fig. 3d, the preponderant bacteria were Proteobacteria（94.29%，77.87%，93.63%，91.18%）and Firmicutes (4.39%, 16.91%, 5.38%, 6.77%) in the ileum of four NFD groups at the levels of the phylum. However, Firmicutes (89.11%) was the preponderant bacteria which about 10-fold higher than Proteobacteria (9.23%) in control group at the levels of the phylum. At species level (Fig. 3e), the preponderant bacteria were *Escherichia_coli* (92.76%, 69.57%, 92.52%,89.38%) in the ileum of NFD groups. But in the control group, the *Lactobacillus_aviarius* (19.32%) and *Escherichia_coli* (8.54%) were the first and second species in ileal microbial communities, respectively. Considering there were violently disruptive changes of microbiota composition between NFD treatment and the control, it is necessary to make further analysis of the microbial function in this study. And the Tax4Fun analysis based on Kyoto Encyclopaedia of Genes and Genomes (KEGG) data predicted differentially expressed functional pathways among groups (Fig. 4).

The four NFD groups harbored very close profiles but rather different from those predicted in the control. As shown in Fig. 4, a higher abundance of functions reported for transporters, secretion system, ABC transporters and two-component system were predicted in NFD groups in comparison to the control, indicating that the microorganism in NFD groups plausibly meets its nutrient requirements for adaption. However, metabolic information related to the DNA repair and recombination protein, the metabolism of amino acids, pyruvate, purine, and pyrimidine appears to be expanded in the control group.

Discussion

There are many methods to determine the endogenous ileum amino acids (IEAAs) losses in broiler chickens, such as the regression method, the enzymatically hydrolyzed casein method, the NFD method and the 15N-leucine single-injection method [6, 11]. Among these various methods, the NFD method is most widely used for its simplicitye, convenience and low cost. However, one of the fundamental concerns of NFD is the absence of dietary protein, and the animals can be expected to suffer from malnutrition due to the deficiency of essential amino acids.

Hence, we sought to investigate the extent to NFD might impact the health of chickens, we used some serum biochemical parameters as an indicator of the basic physiological status and metabolic functions of broiler chickens. The albumin level in the serum of chickens was detected because low albumin levels could serve as a potential marker of poor nutritional status [12]. Besides, most uric acid is produced from the breakdown of dietary and endogenous purines [13], and the correlation analysis data revealed a strong negative relationship between plasma uric acid levels and efficiency of protein retention [14]. In this study, we found that the albumin and uric acid levels were decreased in all the NFD groups as compared with the control, indicating clearly that the protein-deficiency led to malnutrition of animals in NFD treatments. Generally, serum IGF-1 concentration is influenced by nutritional status, particularly protein-energy malnutrition. It has been reported that serum IGF-1 concentration is

reduced in models of severe energy restriction or protein restriction in young growing rats [15]. In this study, the serum IGF-1 concentration in four NFD groups decreased by varying degrees which could reflect some protein restriction. Interestingly, glucose, insulin, and glucagon levels in serum were not influenced by the different ratios of dextrose to starch, suggesting the normal regulation of blood glucose in this study.

Although NFD with different dextrose and starch contents did not alleviate the malnutrition status of broiler chickens, it did change the basic endogenous amino acids of broilers in this study. The most abundant amino acids in the ileal endogenous protein of broilers were Glu, Asp, Thr, Pro, Ser, and Gly [16]. Previous research also showed that the estimate of ileal endogenous flows of total AA was affected when the NFD contained no cornstarch and only dextrose, increased from 12,779 mg/kg of DMI to 17,544 mg/kg of DMI as the proportion of corn starch in NFD decreased from 849.1 to 0 g/kg [9]. Herein, we had further narrowed down the ratio of D/CS in the order of 1, 0.6, 0.33, and 0.14 and found that a higher proportion of dextrose can raise ileal IEAAs flow of broiler chickens with significant changes observed when the ratio of D/CS ranged between 0.6 and 0.33. This could potentially be due to the different contributions of sources of endogenous proteins, such as digestive enzyme secretions, mucus, sloughed epithelial cells and microbial protein.

In the current study, we found the highest proportion of dextrose (D/CS = 1.00) dramatically increases the activity of α-amylase, lipase, sucrase, and maltase. Excessive digestive enzymes might be reused as either a component of other endogenous proteins after degradation and absorption or as recycled enzyme after the conservation process [17]. This might be, in part, result in a high level of IEAAs flow. It is widely believed that glucose absorption takes place by two kinds of transporters, i.e., co-transportation with sodium ions via SGLT1 and facilitated diffusion via GLUT2 [18]. In this study, we found that NFD rich in glucose and starch significantly increased the gene expression of these two glucose transporters, indicating that the chickens adapted to the absorption of high carbohydrates in NFD. It is noteworthy that the digestive enzymatic activity of group C (D/CS = 0.33) resembled closely with the control group, suggesting that perhaps the 0.33 ratio of dextrose to corn starch is appropriate. However, when the proportion was adjusted to 0.15, the dry matter digestibility significantly dropped, which established its unsuitability for the chicken to digest NFD.

In addition to digestive enzymes, mucins also contribute to the IEAAs loss. The mucus layer secreted from goblet cells makes the interface between the gut lumen and the gut epithelium is poorly digested in the small intestine [19]. We observed no significant difference in villi height and crypt depth among the treatment groups, but the number of goblet cells was increased on the villi of jejunum and ileum in NFD treatment groups. Moreover, the gene expression of ileal Mucin 2 was significantly higher than the control when D/CS in NFD were 1.0 and 0.6 ($P < 0.05$). Dietary composition is suggested to influence the differentiation of intestinal epithelial cells, and diets rich in carbohydrates or amino acids lead to the different pattern of cell differentiation [20]. The nourishment of goblet cells to form mucin depends mainly on the absorbed glutamine and glucose, which can be easily provided by prolamines and starch in grain [21]. Previous in vivo studies indicated that feeding starch leads to increased mucus production of goblet cells in pigs and rats [22, 23]. As NFD is rich in starch and dextrose, these results potentially indicate that NFD could promote mucin secretion by increasing the number of goblet cells.

Apart from mucins, microbial protein in the gut is also a contributor to IEAAs. A high-glucose diet has been linked to gut microbial diversity losses where the proportion of Bacteroidetes decreased, while the proportion of Proteobacteria was markedly increased [24]. The literature on the effects of a high sugar diet established its effect on gut microbiota and NFD groups being high in sugar contents directly contributed to modulate the gut microbiota, characterized by a lower Bacteroidetes and a significantly increased proportion of Proteobacteria ($P < 0.05$). Since Proteobacteria with adherent and invasive properties are considered to be a rich source of lipopolysaccharides (LPS), which has been associated with inflammatory bowel disease and metabolic syndrome [27, 28], we speculate that gut microbiome functions may be one variable influencing nutrition metabolism function for animals. We, therefore, used Tax4Fun to predict the functional profile of a microbial community and found that NFD increased pathways corresponding to the genes for transporters, secretion system, and two-component system, which were closely associated with bacterial adaptation. The adaptation of bacterial species not only rely on the release of molecules in the milieu, such as siderophores, exopolysaccharides, and/or protein toxins but also on two-

component system, which allows a pathogen to adapt its gene expression in response to environmental stimuli [27]. Besides, the "secretion systems" facilitate protein toxins transport through the physical barriers that the membranes represent [28]. In this study, the functional prediction of Tax4Fun was consistent with the changes of microbial community, indicating that NFD enriched the pathogenic bacteria survival, which represented a considerable health threat to the chicken intestine.

Conclusion

Overall, this study provides a more comprehensive view of the NFD method and basal IEAAs of broiler chickens. The chickens fed NFD were accompanied by huge digestive physiological alterations, presenting with malnutrition and accumulation of proteobacteria in the gut. Different proportions of dextrose and starch directly affect the basal IEAAs of broiler chickens. A higher proportion of dextrose (D/CS = 1 and 0.6) in NFD increase IEAAs by promoting secretion of digestive enzyme and mucin. But the excessive proportion of starch is unsuitable for the chicken to digest NFD (D/CS = 0.14). Therefore, we suggest the ratio of dextrose to corn starch in NFD at 0.33 might be more appropriate to detect IEAAs of broiler chickens.

Methods

Experimental design, animals, and animal care

The animal care and experimental procedures described in this experiment were conducted according to the Animal Welfare Committee guidelines and had the approval of Ethics committee of Animal Science and Technology College of China Agricultural University (No.AW11059102-1, Beijing, China). And the experiments were performed in accordance with the ARRIVE guidelines (https://arriveguidelines.org).

A total of 210,1-d-old broiler chickens were fed with the same starter diet up to 27-d-old. At 28-d-old, chickens were fasted for 8 hours, then the chickens with similar body weight were allocated to 5 treatment groups in a randomized complete block design for a 3-day trial period to estimate IEAAs losses. The trial was conducted in 5 treatment groups, 6 replicates with 7 chickens per replicate, including a control group (CT, basal diet, normal level of protein) and four NFD groups with different ratios of dextrose to corn starch (D/CS), designated as A (D/CS = 1.00), B (D/CS = 0.60), C (D/CS = 0.33), and D (D/CS = 0.14). Because we detected starch content in basal diet is 38.76%, and we found it is challenging to make a pelleted diet with starch content less than 30% in NFD after the pretest. Therefore, the variation of starch content in NFD is based on 40% in this study to make it feasible to prepare the experimental diet.

All the diets were pelleted by feed granulator (Chengda Machinery Co., Ltd, Laizhou, China) and contained 0.5% titanium dioxide (Sinopharm Chemical Reagent Co., Ltd, Shanghai, China) as an indigestible digestibility marker. The dextrose and corn starch were both purchased from Qinhuangdao Lihua starch Co., Ltd (Qinhuangdao, China), which satisfied the corresponding China National Standard GB/T 8885 and GB/T 20880. The composition of ingredient, the amino acid content of experiment diet was given in Table 1 and Table 2, respectively.

All chickens were raised in the same layer of cages (0.7m^2/per cage), the stocking density was 0.1m^2/per bird from 1d to 31d. The brooding temperature was maintained at 33-35℃ from 1d to 2d, then the temperature dropped by one degree every two days until 21℃. The relative humidity was maintained at 65-70% from 1d to 7d, 50-65% from 8d to 31d. The lighting schedule consisted of 24 h from 1d to 2d, 23h for 3d, 22h for 4d, 21h for 5d, and 20h from 6d to 31d. All birds had free access to feed and water.

No animal deaths occurred during the 3-day trial period.

Sample collection

On day 31, one chicken from each replicate was randomly selected to collect blood was from the brachial vein and subsequently centrifuged at 1,500 × g for 10 min to obtain serum. Afterward, these chicken were euthanatized by injecting pentobarbital sodium (50mg/kg body weight), 1 cm segments of duodenum, jejunum, and ileum were collected in carnoy

fixative (G2312, Solarbio, Japan) for Alcian blue-periodic acid-schiff stain (AB-PAS), the digesta, mucous, and segment of ileum were collected and stored at -80℃. All other chickens were euthanatized with pentobarbital sodium (50 mg/kg BW), intestine removed, and the digesta of distal ileum was collected and mixed, stored in -80℃ overnight then freeze-dried using a vacuum freeze dryer (FD-2, Biocool, Beijing, China).

Calculations of IEAAs and AID

After freeze-drying, the diet and digesta were ground and sifted through a 40-mesh sieve to ensure homogeneity. Dry matter (DM) and 15 amino acids (AA) were analyzed based on the methods illustrated in AOAC International [29]. The AA concentration was measured by Amino Acid Analyzer (A-300, Membrapure, Germany). TiO_2 was determined using the procedure described by Myers et al [30]. The ileal endogenous losses（IEAAs） was calculated from the equation (1), and the AID of DM was calculated using equation (2).

$$(1)\ \text{Ileal endogenous losses of AA (g/kg of DM intake)} = \left(\frac{TiO_{2\ diet}}{TiO_{2\ ileum}}\right) \times AA_{ileum}$$

Where: TiO2 diet and TiO2 ileum represented the TiO2 concentrations (g/kg of DM) in the NFD and ileal digesta from chickens fed the NFD, respectively. The AA ileum represented the AA concentrations (g/kg) in the ileal digesta from the chickens.

$$(2)\ \text{AID of DM (\%)} = \left[1 - \left(\frac{TiO_{2\ diet}}{TiO_{2\ ileum}}\right) \times \left(\frac{DM_{ileum}}{DM_{diet}}\right)\right] \times 100\%$$

Where: TiO2 diet and TiO2 ileum represented the TiO2 concentrations (g/kg) in the diets and ileal digesta from chickens, respectively; DM diet and DM ileum represented the DM concentrations (g/kg) in the diets and ileal digesta from the chickens, respectively.

Serum metabolites

The glucose, total protein (TP), albumin, and uric acid were determined by an automated biochemical analyzer (TBA-120FR, TOSHIBA, Japan). The insulin-like growth factors -1(IGF-1) was detected by IGF-1 600 ELISA kit (DRG, ELA-4140, Germany). The glucagon (GLUN) and insulin (INS), were detected by automatic radioimmunocounter (XH-6080, Xi'an Nuclear instrument Factory, China).

Intestinal morphology

The tissue sections and AB-PAS stain of duodenum, jejunum, and ileum of broiler chickens were prepared by Servicebio Co., Ltd (Beijing, China). The intestinal morphology was measured based on 8 representative complete villi in the same AB-PAS stained slide. Mucosal villus height was defined as the length from the tip of the villus to the crypt opening and the associated crypt depth was determined from the crypt opening to the crypt base. The number of goblet cells was quantified by counting the number of stained goblet cells per 100 um length of villus and present as the average number of goblet cells per 10 intestinal villi.

The activity of the digestive enzyme

The mucosal tissue (0.2 gram) was homogenized (4000 rpm, 10 min) with an Ultra-Turrax homogenizer (JIUPIN-92, JIUPIN, WuXi, China) in 6 volumes of saline (4°C) to collect the homogenate. According to the instructions, the activities of sucrose and maltase were measured (n=6) using commercial assay kits (A082-21, A082-31, Nanjing Jiancheng Bioengineering Institute, Nanjing, China). And the data were collected by optical density (all 505nm) measurement on a microplate reader (SpectraMax i3x, Molecular Devices, LLC, USA).

The scraped intestinal digesta and mucosal samples were immediately snap-frozen using liquid nitrogen. Then, the intestinal digesta (0.2 gram) was homogenized (3500 rpm, 10 min) with an Ultra-Turrax homogenizer (JIUPIN-92, JIUPIN, WuXi, China) in 9 volumes of saline (4°C) to collect the homogenate. And the amylase, chymotrypsin, lipase activity was determined according to the instruction of kit (C016-1-1, A080-3-1, A054-2-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China). And the data were then collected using a spectrophotometer (Ultrospec 2100 pro, Amersham Biosciences, USA).

Microflora analysis of digesta in ileum

DNA extraction was performed using a QIAampTM Fast DNA Stool Mini Kit (Qiagene, No. 51604). High-throughput sequencing of 16S rDNA gene amplicons was performed by Novogene Biotech Co., Ltd. (Beijng, China) using a NovaSeq PE250 platform (Novogene Biotech Co., Ltd, Beijng, China). The high-quality sequences were clustered into operational taxonomic units (OTUs) at a 97% similarity level, and a total of 1808 OTUs were obtained. Then the OTUs sequences were annotated with Silva132 database. According to the species annotation, the α diversity and β diversity were further calculated, and the differences between groups were compared to reveal the different characteristics of microbial community structure under different treatments.

Gene expression

Total RNA was isolated from jejunum and ileum using RNA Easy Fast Tissue/Cell Kit (DP451, Tiangen Biotech Co.,Ltd, Beijing, China) , and the concentration and purity of each sample were determined at 260/280nm. Then, 1ug of total RNA was reversed into the first-strand cDNA using a kit (RR047A, Takara, Kyoto, Japan). Real-time PCR of mRNA was conducted using the ABI 7500 Fluorescent Quantitative PCR system (Applied Biosystems, Bedford, MA). Each RT reaction was carried out by an SYBR Premix ExTaq kit (RR420A, Takara, Kyoto, Japan). The gene-specific primers were commercially manufactured (Table 3; Sangon Biotech, Shanghai, China), and β-actin was chosen as the house-keeping gene. The relative gene expression levels were calculated by the method [31]. In addition, the protocol of melting curve analysis set as follows: 95°C for 30sec; 40 cycles of 95°C for 5sec and 60°C for 34 sec; 15sec for 95°C, 1min for 60°C and 15sec for 95°C.

Statistical Analysis

The data was analysed by SPSS, version 20.0 (SPSS, IBM, Chicago, IL, USA). Data distribution were checked by Shapiro-Wilk test. Normally distributed data were analysed by one-way ANOVA for comparisons among groups, and then followed by the Dunnett's post hoc test. Data were displayed as means value ± standard deviation, and the standard error of mean (SEM) are represented for all pooled data. P value less than 0.05 were considered statistically significant, and P value less than 0.01 indicates extremely significant differences.

Declarations

Acknowledgements

We would like to thank Ping Lu, Kejun Cao, and Zuquan Pi for technical advice and the assistance with animal experiments.

Contributions

The experimental scheme was designed by Z,HJ.and Y.JM. Besides, W.W., X,YW., C,YH. and W,YL. participated in the experiment process and assisted in sampling. Z,HJ. analyzed data and wrote the paper, Y,JM. and T.M. provided the necessary experimental equipment and key guidance during the experiment process. The authors read and approved the final manuscript.

Ethics declarations

Competing interests

The authors declare no competing financial interest.

Funding

This research was supported by the National Natural Science Foundation of China

(No. 31772620) and the System for Poultry Production Technology, Beijing Agriculture Innovation Consortium (Project Number: BAIC04–2019).

Corresponding author

Correspondence to Jianmin Yuan, Email: yuanjm@cau.edu.cn

References

1. Kong, C. & Adeola, O. Comparative amino acid digestibility for broiler chickens and White Pekin ducks. *Poult. Sci.* 92, 2367-74 (2013).
2. Jansman, A.J.M., Smink, W., van Leeuwen, P. & Rademacher, M. Evaluation through literature data of the amount and amino acid composition of basal endogenous crude protein at the terminal ileum of pigs. *Anim. Feed. Sci. Tech.* 98, 49-60 (2002).
3. Park, C.S., Ragland, D., Helmbrecht, A., Htoo, J.K. & Adeola, O. Digestibility of amino acid in full-fat canola seeds, canola meal, and canola expellers fed to broiler chickens and pigs. *J Anim Sci.* 97,803-812 (2019).
4. Adedokun, S.A., Parsons, C.M., Lilburn, M.S., Adeola, O. & Applegate, T.J. Comparison of ileal endogenous amino acid flows in broiler chicks and turkey poults. *Poult Sci.* 86,1682-1689 (2007).
5. Golian, A., Guenter, W., Hoehler, D., Jahanian, H. & Nyachoti C.M. Comparison of various methods for endogenous ileal amino acid flow determination in broiler chickens. *Poult. Sci.* 87,706-712 (2008).
6. Adedokun, S.A., Jaynes, P., El-Hack, M.E.A., Payne, R.L. & Applegate, T.J. Standardized ileal amino acid digestibility of meat and bone meal and soybean meal in laying hens and broilers. *Poult. Sci.*93, 420-428 (2014).
7. Lu, P.Y., Wang, J., Wu, S.G., Gao, J., Dong, Y., Zhang, H.J. & Qi, G.H. Standardized ileal digestible amino acid and metabolizable energy content of wheat from different origins and the effect of exogenous xylanase on their determination in broilers. *Poult. Sci.* 99, 992-1000 (2020).
8. Kong, C. & Adeola, O. Ileal endogenous amino acid flow response to nitrogen-free diets with differing ratios of corn starch to dextrose in broiler chickens. *Poult. Sci.* 92,1276-1282 (2013).
9. Adedokun, S.A., Pescatore, A.J., Ford, M.J., Jacob, J.P. & Helmbrecht, A. Examining the effect of dietary electrolyte balance, energy source, and length of feeding of nitrogen-free diets on ileal endogenous amino acid losses in broilers. *Poult. Sci.* 96, 3351-3360 (2017).
10. Hu, R., Li, J., Soomro, R.N., Wang, F., Feng, Y., Yang, X. & Yao, J.H. The 15N-leucine single-injection method allows for determining endogenous losses and true digestibility of amino acids in cecectomized roosters. *Plos. One.*12, e188525 (2017).
11. Kim, S., McClave, S.A., Martindale, R.G., Miller, K.R. & Hurt, R.T. Hypoalbuminemia and Clinical Outcomes: What is the Mechanism behind the Relationship? *Am. Surg.* 83, 1220-1227 (2017).
12. Bailey, C.J. Uric acid and the cardio-renal effects of SGLT2 inhibitors. *Diabetes. Obes. Metab.*21, 1291-1298 (2019).
13. Swennen, Q., Janssens, G.P., Millet, S., Vansant. G., Decuypere, E. and Buyse, J. Effects of substitution between fat and protein on feed intake and its regulatory mechanisms in broiler chickens: endocrine functioning and intermediary metabolism. *Poult. Sci.* 84, 1051-1057 (2005).
14. Prewitt, T.E., D'Ercole, A.J., Switzer, B.R. & Van Wyk, J.J. Relationship of serum immunoreactive somatomedin-C to dietary protein and energy in growing rats. *J. Nutr.* 112, 144-150 (1982).
15. Ravindran, V. Progress in ileal endogenous amino acid flow research in poultry. J *Anim. Sci. Biotechno.* 12, 5 (2021).
16. Rothman, S., Liebow, C. & Isenman, L. Conservation of digestive enzymes. *Physiol. Rev.* 82, 1-18 (2002).

17. Qi, X. & Tester, R.F. Fructose, galactose and glucose–In health and disease. *Clin. Nutr. ESPEN.* 33,18-28 (2019).
18. Montagne, L., Piel, C. & Lallès, J.P. Effect of diet on mucin kinetics and composition: nutrition and health implications. *Nutr. Rev.* 62, 105-114 (2004).
19. Moor, A.E. & Itzkovitz, S. Spatial transcriptomics: paving the way for tissue-level systems biology. *Curr. Opin. Biotechnol.* 46, 126-133 (2017).
20. Moran, E.T. Gastric digestion of protein through pancreozyme action optimizes intestinal forms for absorption, mucin formation and villus integrity. *Anim. Feed. Sci. Tech.* 221, 284-303 (2016).
21. Mentschel, J. & Claus, R. Increased butyrate formation in the pig colon by feeding raw potato starch leads to a reduction of colonocyte apoptosis and a shift to the stem cell compartment. *Metabolism*. 52, 1400-1405 (2003).
22. Morita, T., Tanabe. H., Takahashi, K. & Sugiyama, K. Ingestion of resistant starch protects endotoxin influx from the intestinal tract and reduces D-galactosamine-induced liver injury in rats. *J. Gastroenterol. Hepatol.* 19, 303-313 (2004).
23. Moon, H.D., Lee, E., Oh, M.J., Kim, Y. & Park, H.Y. High-glucose or -fructose diet cause changes of the gut microbiota and metabolic disorders in mice without body weight change. *Nutrients*.10, 761 (2018).
24. Mukhopadhya, I., Hansen, R., El-Omar, E.M. & Hold, G.L. IBD-what role do Proteobacteria play? Nat Rev Gastroenterol Hepatol. 9, 219-230 (2012).
25. Shin, N.R., Whon, T.W. & Bae, J.W. Proteobacteria: microbial signature of dysbiosis in gut microbiota. *Trends. Biotechnol.* 33, 496-503 (2015).
26. Dahyot, S., Oxaran, V., Niepceron. M., Dupart, E., Legris, S., Destruel, L., Didi, J., Clamens, T., Lesouhaitier, O., Zerdoumi, Y., Flaman, J.M. & Martine, P.C. Role of the LytSR two-component regulatory system in staphylococcus lugdunensis biofilm formation and pathogenesis. *Front. Microbiol.* 11, 39 (2020).
27. Journet, L. & Cascales, E. The type VI secretion system in escherichia coli and related species. *Ecosal. Plus.* 7,1-20 (2016).
28. Horwitz, W. & Latimer, G. Official methods of analysis of AOAC International. 18th ed. Gaithersburg, MD, USA: AOAC International (2005).
29. Myers, W.D., Ludden, P.A., Nayigihugu, V. & Hess, B.W. Technical Note: A procedure for the preparation and quantitative analysis of samples for titanium dioxide1. *J. Anim. Sci.* 82, 179-183 (2004).
30. Livak, K.J. & Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods.* 25, 402-408 (2001).

Tables

Table 1 Composition and nutrient levels of nitrogen-free diet and basal diets (%)

Group	A	B	C	D	CT[1)]
Zeolite [2)]	8.88	8.88	8.88	8.88	0
Dextrose	40	30	20	10	0
Corn starch	40	50	60	70	0
Cellulose[3)]	4	4	4	4	0
Soybean oil	3	3	3	3	3
$CaHPO_4$	1.9	1.9	1.9	1.9	1.9
Limestone	1	1	1	1	1
NaCl	0.3	0.3	0.3	0.3	0.3
Choline chloride	0.2	0.2	0.2	0.2	0.2
Multi-vitamin[4)]	0.02	0.02	0.02	0.02	0.02
Multi-mineral[5)]	0.2	0.2	0.2	0.2	0.2
TiO_2 [6)]	0.5	0.5	0.5	0.5	0.5
Corn（7.5% CP）	0	0	0	0	54.43
Soybean meal（46% CP）	0	0	0	0	38.05
DL-Met	0	0	0	0	0.2
L-Lys·HCL	0	0	0	0	0.2
Total（%）	100	100	100	100	100

[1)] Nutrient level: Metabolizable energy 2.95MC/kg, Crude protein 22.50%, Ca 1%, non-phytate phosphorous 0.45%.

[2)] The zeolite is harmless and indigestible to animals, it is suitable for animal feed application (Deheng mineral products Co., Ltd, Shijiazhuang, China). We use the zeolite as premix carrier in the NFD diets, because it contains almost no protein, energy or any other digestible nutrients.

[3)] Sodium carboxymethyl cellulose (CAS: 9004-32-4, Sinopharm Chemical Reagent Co., Ltd, Shanghai China).

[4)] Per kg of diet: Vitamin A 10800 IU, Vitamin D3 2160 IU, Vitamin E 4.6 mg, Vitamin K3 1.0 mg, Vitamin B1 0.4 mg, Vitamin B2 5 mg, Vitamin B12 6 mg, folic acid 0.1 mg, niacin 7 mg, pantothenic acid 5 mg.

[5)] Per kg of diet: Cu 6mg, Zn 50mg, Fe 60mg, Fe 0.15mg, I 0.35mg.

[6)] TiO_2: (CAS: 13463-67-7, Sinopharm Chemical Reagent Co., Ltd, Shanghai China).

Table 2 The amino acid contents of the experimental diets (DM basis)

AA (g/kg)	A	B	C	D	CT
Asp	0.09	0.11	0.13	0.15	21.61
Thr	0.03	0.05	0.07	0.08	7.13
Ser	0.04	0.04	0.07	0.09	10.99
Glu	0.04	0.05	0.06	0.08	35.90
Gly	0.04	0.05	0.07	0.08	8.63
Ala	0.13	0.16	0.20	0.23	9.80
Val	0.09	0.11	0.13	0.15	7.87
Ile	0.04	0.05	0.07	0.08	7.96
Leu	0.13	0.16	0.20	0.23	15.96
Tyr	0.04	0.05	0.07	0.08	7.05
Phe	0.09	0.11	0.13	0.15	11.27
His	0.00	0.00	0.00	0.00	4.92
Lys	0.04	0.05	0.07	0.08	12.77
Arg	0.04	0.05	0.07	0.08	13.80
Pro	0.09	0.11	0.13	0.15	12.09

Group CT was fed basal diet, group A-D were fed NFD with varying ratios of dextrose to corn starch investigated were 1, 0.6, 0.33, and 0.14 in groups A, B, C and D, respectively.

Table 3 Nucleotide sequence of primers for gene expression analysis

Target gene	F:forward, R: reverse	Primer sequence (5'→3')	Accession no.	Size (bp)
β-actin	F	TGTTACCAACACCCACACCC	NM_205518	110
	R	TCCTGAGTCAAGCGCCAAAA		
SGLT1	F	CATCTTCCGAGATGCTGTCA	XM_015275173	169
	R	CAGGTATCCGCACATCACAC		
GLUT2	F	CCGCAGAAGGTGATAGAAGC	NM_207178	87
	R	ATTGTCCCTGGAGGTGTT		
Mucin2	F	TCACCCTGCATGGATACTTGCTCA	NM_001318434.1	228
	R	TGTCCATCTGCCTGAATCACAGGT		

SGLT-1: Na(+)-glucose cotransporter 1;GLUT-2: Glucose transporter type 2

Table 4 The effects of different nitrogen-free diet on serum biochemical parameters of broiler chickens

Item	A	B	C	D	CT	SEM	*P*-value
Glucose （mmol/L）	12.59±3.48	13.59±1.16	12.19±1.52	12.58±4.79	13.20±2.33	0.930	0.548
Insulin （IU/ml）	5.72±1.06	6.45±1.19	7.36±1.06	6.73±1.58	6.98±0.99	0.218	0.206
Glucagon （pg/ml）	159.20±32.67	151.44±44.10	156.37±38.95	143.54±28.20	133.18±22.37	5.862	0.686
IGF-1 （ng/ml）	16.26±2.14^{c}	16.86±3.26^{bc}	19.04±1.97^{ab}	15.47±0.88^{c}	21.04±0.16^{a}	1.019	0.008
TP（g/L）	24.10±3.47	23.75±3.10	23.83±2.37	22.47±3.65	21.32±2.09	0.548	0.466
Albumin （g/L）	9.10±1.14^{b}	8.42±0.83^{b}	9.53±0.96^{b}	8.88±1.17^{b}	13.10±1.53^{a}	0.210	<0.001
Uric acid （umol/L）	143.67±50.95^{b}	134.33±35.52^{b}	154.83±22.68^{b}	130.17±42.65^{b}	254.33±51.71^{a}	7.719	<0.001

IGF-1: Insulin-like growth factor-1; TP: Total protein.

Group CT was fed basal diet, group A-D were fed NFD with varying ratios of dextrose to corn starch investigated were 1, 0.6, 0.33, and 0.14 in groups A, B, C and D, respectively (n=6).

Table 5 The loss of basic IEAAs (mg/kg DM intake) in ileum of broiler chickens

AA	A	B	C	D	SEM	*P*-value
Asp	1105.69±187.64^{a}	1134.16±190.44^{a}	798.63±96.15^{b}	761.44±87.22^{b}	30.333	<0.001
Thr	1119.03±163.92^{a}	1030.45±104.13^{a}	768.19±102.18^{b}	619.97±112.82^{b}	25.183	<0.001
Ser	1223.17±238.66^{a}	1270.32±160.74^{a}	930.74±121.55^{b}	826.61±105.81^{b}	33.660	<0.001
Glu	2157.91±524.04^{a}	2064.10±323.47^{a}	1446.99±235.24^{b}	1309.45±189.08^{b}	69.990	<0.001
Gly	614.34±166.69^{a}	622.25±99.78^{a}	437.90±46.94^{b}	408.56±62.41^{b}	56.650	<0.001
Ala	569.36±161.92^{a}	526.32±76.94^{a}	307.51±89.00^{b}	293.90±49.50^{b}	21.043	<0.001
Val	636.56±160.67^{a}	635.08±86.52^{a}	438.81±52.06^{b}	436.92±53.04^{b}	57.146	0.001
Ile	431.24±127.76^{a}	462.02±77.13^{a}	333.66±52.75^{b}	308.96±30.64^{b}	16.455	0.009
Leu	812.24±188.95^{a}	861.50±140.88^{a}	614.58±100.67^{b}	580.35±59.44^{b}	26.852	0.002
Tyr	478.95±104.36^{a}	491.37±76.48^{a}	341.95±54.23^{b}	330.09±35.45^{b}	14.768	0.001
Phe	455.69±145.76^{ab}	560.67±96.53^{a}	393.36±58.36^{b}	357.55±82.74^{b}	20.619	0.013
His	733.22±82.43^{a}	745.10±32.46^{a}	557.33±56.84^{c}	645.94.94±60.66^{b}	14.189	<0.001
Lys	596.30±227.65^{a}	580.71±147.92^{ab}	427.80±50.81^{ab}	411.19±53.55^{b}	49.008	0.060
Arg	557.96±187.82^{a}	549.30±121.32^{a}	379.51±54.39^{b}	323.75±57.98^{b}	59.439	0.004
Pro	690.00±190.16^{a}	656.65±98.04^{a}	459.27±162.83^{b}	476.17±131.53^{b}	30.549	0.025

Group CT was fed basal diet, group A-D were fed NFD with varying ratios of dextrose to corn starch investigated were 1, 0.6, 0.33, and 0.14 in groups A, B, C and D, respectively (n=6).

Table 6 The effects of nitrogen-free diet on intestinal morphology of broiler chickens

	Item	A	B	C	D	CT	SEM	*P*-value
Duodenum	Villus height（um）	1631.70	1538.99	1550.39	1573.12	1435.82	36.12	0.550
	Crypt depth（um）	154.26	166.27	58.19	167.74	174.34	3.828	0.497
	V/C[1)]	10.76	9.40	9.96	9.61	8.48	0.235	0.067
	Number of goblet cells （cells/100um）[2)]	7	9	7	8	8	0.93	0.331
Jejunum	Villus height（um）	1123.91	1209.70	1100.90	1188.18	1105.83	25.040	0.534
	Crypt depth（um）	135.98	159.61	143.83	145.81	161.31	3.712	0.268
	V/C	8.50	7.91	7.78	8.34	7.02	0.187	0.152
	Number of goblet cells （cells/100um）	11^{a}	12^{a}	10^{a}	10^{a}	8^{b}	0.227	0.001
Ileum	Villus height（um）	661.03	800.76	647.64	684.60	644.40	19.992	0.097
	Crypt depth（um）	122.60	133.27	125.27	127.65	146.92	2.500	0.077
	V/C	5.46^{a}	6.06^{a}	5.26^{a}	5.47^{a}	4.47^{b}	0.112	0.008
	Number of goblet cells （cells/100um）	11^{a}	12^{a}	12^{a}	13^{a}	9^{b}	0.277	0.022

[1)]V/C: The ratio of villus height to crypt depth.

[2)]The number of goblet cells were quantified by counting the number of stained goblet cells per 100 um length of villus, and present as the average number of goblet cells per 8 intestinal villi.

Group CT was fed basal diet, group A-D were fed NFD with varying ratios of dextrose to corn starch investigated were 1, 0.6, 0.33, and 0.14 in groups A, B, C and D, respectively (n=6).

Table 7 The ileal digestive enzyme activity of broiler chickens（U/mg prot）

Item	A	B	C	D	CT	*P*-value
α-amylase[1)]	889.28 ± 117.92^{a}	733.27 ± 137.42^{ab}	756.85 ± 199.79^{ab}	648.04 ± 86.44^{b}	618.71 ± 78.68^{b}	0.021
Lipase[1)]	138.42 ± 60.91^{a}	50.80 ± 32.66^{b}	43.15 ± 29.45^{b}	33.64 ± 22.17^{b}	79.66 ± 24.98^{b}	<0.001
Chymotrypsin[1)]	13.99 ± 5.57^{b}	45.61 ± 29.63^{a}	21.30 ± 11.37^{ab}	47.36 ± 26.49^{a}	16.98 ± 14.15^{b}	0.029
Sucrase[2)]	52.37 ± 20.98^{a}	23.70 ± 13.15^{bc}	28.00 ± 17.70^{b}	6.69 ± 3.66^{c}	9.98 ± 6.98^{bc}	<0.001
Maltase[2)]	889.28 ± 117.92^{a}	733.27 ± 137.42^{ab}	756.85 ± 199.79^{ab}	648.04 ± 86.44^{b}	618.71 ± 78.68^{b}	0.021

[1)]The enzyme activity in the digesta of ileum (n=6).

[2)]The enzyme activity in the mucous of ileum (n=6).

Group CT was fed basal diet, group A-D were fed NFD with varying ratios of dextrose to corn starch investigated were 1, 0.6, 0.33, and 0.14 in groups A, B, C and D, respectively.

Figures

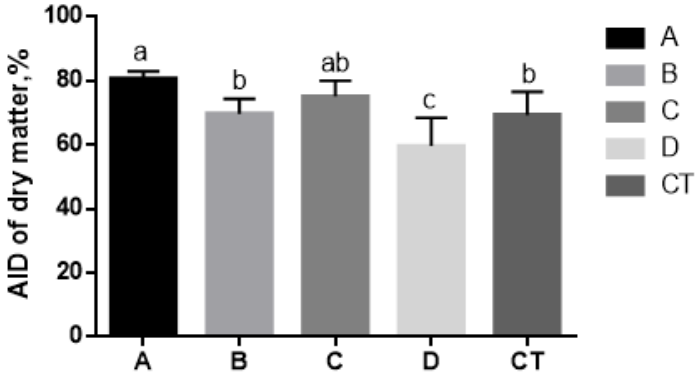

Figure 1

The AID of dry matter in the ileum of broiler chickens

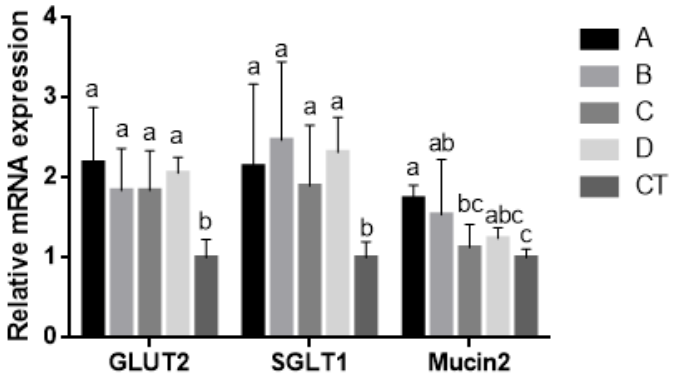

Figure 2

The effects of NFD on the relative gene expression of GLUT2, SGLT1 and Mucin2.

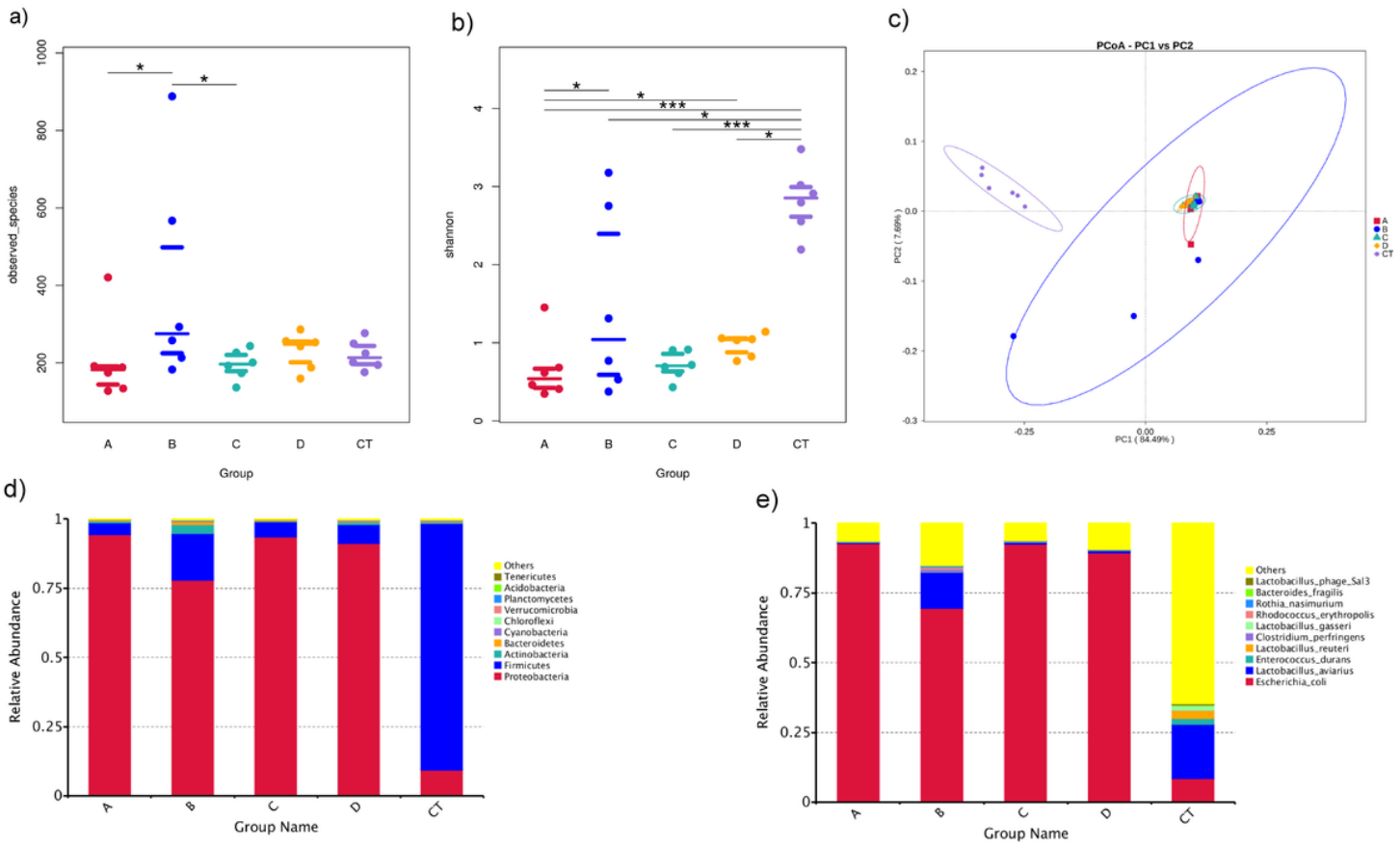

Figure 3

The analysis of alpha and beta diversity of microbial community composition in ileum of broiler chickens (n=6)

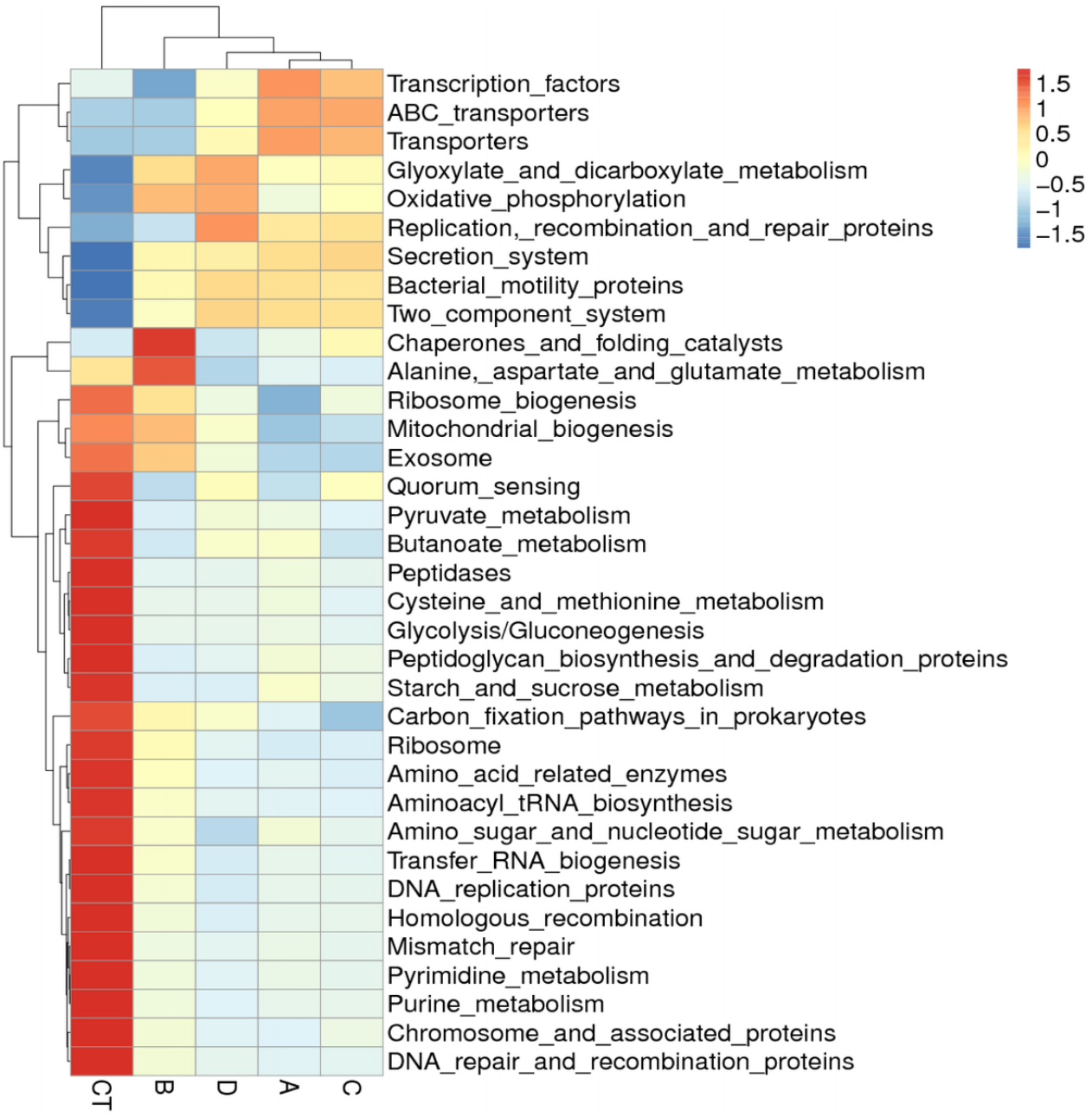

Figure 4

Cluster analysis of the functional relative abundance of Tax4Fun