Effect of fermented feed additive on the broilers' histology/morphology of the GIT, immune status, and microbiota

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Abstract

This study was aimed to analyse the effect of a fermented feed additive on the broilers' histology/morphology of the GIT, immune status, and microbiota as an alternative to AGP. Four groups of 288-day-old male and female Arbor Acre broilers were randomly divided (6 replicates in each group each with 12 chicken). Negative control (NC; basal diet), positive control (PC; basal diet +antibiotic virginiamycin 15ppm) one of the 2 fermented feed additives (FFA) diets prepared by adding, fermented feed additive low dose (FFL; basal diet + 0.3kg/t *Lactobacillus casei* fermented feed additive) and fermented feed additive high dose (FFH; 3kg/t *Lactobacillus casei* fermented feed additive). Composition of fermented feed: total bacteria count: 2×10^9 CFU/g Lactic acid 20mg/g, acetic acid 5mg/g, succinic acid 7mg/g, pyruvic acid 0.1mg/g. The research demonstrated that including fermented feed additives (FFA) in the diet provided overall benefits, FFL and PC diets showed improved (1-21 day) feed conversion ratio (FCR) compared to NC from 0-42 day ($P < 0.05$). FFH and FFL showed improve intestinal morphology within the duodenum, jejunum, and ileum with higher villus height (VH), and villus height to crypt ratio (VH/CR) ($P < 0.05$). Duodenum and ilium crypt depth was highest in PC and FFL respectively ($P < 0.05$). FFH had a significantly lower thymus weight compared to NC ($P < 0.05$). Furthermore, fermented feeds FFL and FFH influenced the intestinal immunity-related mRNA levels and increased expression of IL-10 ($P < 0.05$). FFL down-regulated the mRNA expressions of IL-1 β and TLR4 ($P < 0.05$). Both fermented feeds FFL and FFH positively modulated the composition and variety of cecal microbiota compared to the NC.

Introduction

Previously, fermentation has been associated with methods that improve the nutritional value of unconventional feed ingredients for broilers. In recent decades, the fermentation process has been employed to produce functional feeds that have the potential to improve histology/morphology of the GIT, immune status, and microbiota status of broiler. After banned from antibiotic growth promoter (AGP), the demand for alternatives in poultry was increased due to the higher demand for antibiotic-free poultry (Yang et al. 2009). Therefore, reducing antibiotic usage and finding other alternatives for antibiotics is an unmet need for animals and human health (Stanton 2013; Wang et al. 2015). Several alternatives were introduced to the market include degrading enzymes, yeast extracts, plant extracts, fermented feed, prebiotics, symbiotics, and probiotics (Aktas et al. 2015; Ivory et al. 2008; Sopková et al. 2017). Fermented feed and probiotics have been evaluated as technically viable substitutes to antibiotics in broiler feed, fermented feeds shown to have growth-promoting effects (Yeh et al. 2018), and improve the palatability of feedstuff, and have positive effects on nutrients utilization and reduce the greenhouse gases (GHG) emission (Gao et al. 2020).

Solid-state fermentation (SSF) is one of the oldest fermentation methods, it mimics the natural environment of the microorganism, it contains a solid matrix that serves both functions: as the source and provides nutrients for microbial growth with a little or no free water (Singhanian et al. 2017). Generally,

in countries such as China, mainly SSF technique is used to neutralize the undesirable effect of [anti-nutritional factors](#) in certain feed ingredients (Wang et al. 2014).

Nutrient absorption, nutrient digestibility, and metabolism have all been linked to the gut microbiota, and thus animal productivity is influenced by microbial composition and diversity; additionally, diet, including feed components and feed additives, can modulate gut microbial composition and diversity (Zhang et al. 2016b). Fermented feed also positively modulated the gut microbiota (Alshelmani et al. 2016; Zhang et al. 2016a), such as hens fed a diet supplemented with *Lactobacillus* as starter cultures decrease *Enterobacteriaceae* fecal counts (Loh et al. 2007), while fermented moist feed reduced the number of *Coliforms* and *Streptococci* within the broilers' small intestine (Missotten et al. 2013). Several studies have been shown that *L. casei* strains can alter the GIT microbiota and affect the immune response of the host (Aktas et al. 2015; Ivory et al. 2008). Whereas, the broiler GIT microbiota contains hundreds of bacterial species, making traditional culture-based approaches difficult to cultivate and study the composition, diversity, and structure of intestinal microbiota. The 16s rDNA gene sequence has been widely used in recent years in a variety of studies to investigate microbial composition and diversity, as well as their relationship to growth performance (Li et al. 2019) and immunity of animals (Aktas et al. 2015). To the best of our knowledge, there is currently very little information on the effects of fermented feed on cecal microbial composition and diversity in broilers.

The poultry industry thus concentrates on making nutrition more efficient in order to help the alternate of AGP. Based on the evidence presented above, we hypothesize that the use of FFA in broiler chicken diets may modulate cecal microbiota and consequently without affecting growth performance parameters. The present study assumes that FFA can improve the growth performance and immunity. Hence, the present study aimed to test the fermented feed supplementation is linked with improving growth performance, immunity, and compositional or functional changes in the chickens' GIT microbiome.

Materials And Methods

South China Agricultural University's Animal Care and Use Committee reviewed and approved the protocol (Project number 2019B020218001). All procedures were carried out in accordance with the guidelines in the Ministry of Science and Technology's Guide for Experimental Animals (Guangzhou, China), and every effort was made to minimize suffering.

Preparation of fermented feed

Our laboratory screened the *Lactobacillus casei* (C37M41) used in this study. *Lactobacillus casei* (C37M41) was cultured in de Man, Rogosa, and Sharp (MRS) liquid medium at 37 °C for 18 h with shaking at 200 rpm. The basal substrate included corn, soybean meal, and wheat bran in 6:2:2 respectively, 5% yeast extract, 5% molasses were also mixed and poured into a 500 mL Erlenmeyer flask with a glass stopper, supplemented with sterile water to achieve a moisture content of 40%. Inoculation of the wet mixed substrate with *Lactobacillus casei* (C37M41) (1×10^9 cfu/g) and fermented at 37 °C for 24

h. The fermented mixture was placed in a one-way valve plastic bag (Rou Duoduo Biotechnology Co., Beijing, China) after the first stage of fermentation, inoculated with *Lactobacillus casei* (1×10^9 cfu/g), and incubated at 37 °C in anaerobic conditions (the second-stage of fermentation). Controls were inoculated flasks. All of the experimental procedures for uninoculated samples were the identical as for inoculated feed, with the exception of the addition of sterile medium (LB and MRS) rather than of inoculated bacteria. Triplicates of inoculated and uninoculated samples (control) were set up. The inoculated feed fermented for 14 days at 37 °C under anaerobic conditions. Fermented feed was added to the basal diet, mixed evenly, and then made into pellets. The main content in the fermented feed was total bacteria count 2×10^9 cfu/g, lactic acid 20 mg/g, acetic acid 5 mg/g, succinic acid 7 mg/g, and pyruvate 0.1 mg/g.

Birds, Diets, and Experimental Design

The broiler chicks were managed in accordance with Arbor Acre broiler chicken guidelines. The test diet was formulated concerning NRC (1994) and NY/T 33-2004 and combined with the AA broiler feeding manual. Four groups of 288-day-old male and female broiler chickens were divided at random (6 replicates in each group each with 12 chicken). Negative control (NC; basal diet), Positive control (PC; basal diet +antibiotic virginiamycin 15ppm) one of the 2 fermented feed additives (FFA) diets prepared by adding, fermented feed additive low dose (FFL; basal diet + 0.3kg/t *L. casei* fermented feed additive) and fermented feed additive high dose (FFH; 3kg/t *L. casei* fermented feed additive). Broiler chicks were fed and watered *ad libitum*, and a 243L:1D light regime was maintained. The experiment lasted 42 days, divided into a starter period (0 to 21 day) and a finisher period (22 to 42 day). The composition and nutrient content of the basic diet is shown in Table 1. The experimental feed was pelletized and fed in the form of pellets.

Growth Performance

During the experiment, mortality was recorded daily. On the 21st and 42nd day, body weight (BW) and feed intake were recorded. The average daily feed intake (**ADFI**), average daily gain (**ADG**), and feed conversion ratio (**FCR**) were calculated.

2.4 Sampling Procedure

On the 21st and 42nd day, one chicken was randomly selected from each replication, to obtain serum samples, blood samples were collected before slaughtering and centrifuged, which were then stored at – 20°C for further study. Body weight was recorded after a 12 h feed withdrawal and sacrificed.

After bleeding and defeathering, full eviscerated weight was measured when all the internal organs were excised (head, neck, claws, trachea, esophagus, stomach, gizzard, gizzard contents, intestines, spleen, pancreas heart, liver, glandular, abdominal fat, full craw and cutin membrane, and gonads). At 30 minutes postmortem, the whole thigh and breast muscle tissue were removed from the animal carcasses. The abdominal fat, thigh muscle and breast muscle (including all of fat visceral region and abdominal cavity)

were weighed, as a percentage of body weight, the weight percentage of fully eviscerated yield was calculated. Breast muscle, thigh muscle, abdominal fat, and liver weight percentages were calculated as a percentage of the weight of the eviscerated carcass.

The thymus, bursa, and spleen were immediately removed, dried, and individually weighed (g) for each individual, and the ratio of thymus, bursa, and spleen weight: body weight (%) was calculated, where $\text{organ index} = (\text{organ weight in grams}) / (\text{live weight, in grams}) \times 100$. For the expression analysis of inflammatory factors middle section of the jejunum and ileum tissue were placed in a 2 mL centrifuge tube, and then quickly frozen in liquid nitrogen and transferred to an ultra-low temperature refrigerator at -80°C . For intestinal morphology, the duodenum, jejunum, and ileum were collected 2 cm each and placed in a 10% middle (PH=7.4) fixed in formalin fixative and stored in a refrigerator at 4°C . Cecal contents were placed in a 1.5 mL centrifuge tube, quickly frozen in liquid nitrogen, and then transferred to an ultra-low temperature refrigerator at -80°C for storage.

Inflammatory Factors in the Intestine

RNA isolation

Chicken intestinal tissues were collected, and RNA was extracted according to the manufacturer's instructions. RNA was then purified using the RNEasy Mini kit (QIAGEN), where tissues were homogenized in 600 ml of RLT buffer With Tissue Ruptor that is a handheld rotor-stator homogenizer (Qiagen Inc.), as well as total RNA was eluted in 50 μl of DNase-free water before being stored at -80°C . A spectrophotometer was used to measure the amount of RNA (NanoDrop Products, Wilmington, DE). Each sample's total RNA (300 ng) was extracted. The Primer Express software program was used to design the probe and primer set for the 28S rRNA and cytokines (PE Applied Biosystems, Foster City, CA). A well-designated technique was used to quantify chemokine and cytokine mRNA expression. Probes and primers for chemokines and cytokines are listed in Table 2. One-step RT-PCR master mix reagents and TaqMan fast universal PCR master mix were used for the qRT-PCR. The Applied Biosystems 7500 Fast real-time PCR system was used for amplification and detection of specific products, with the following cycle profile: one cycle of 48°C for 30 min and 95°C for 20 s followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. The enhanced fluorescence found by the 7500 Fast sequence detection system during PCR amplification was due to hydrolysis of the target-specific probes by the rTth DNA polymerase's 5' nuclease activity. Normalization was carried out on the housekeeping gene 28S rRNA. For each sample, the correction factor was calculated by dividing the mean threshold cycle (CT) value for the 28S rRNA-specific product in that sample by the overall mean CT value for the 28S rRNA-specific product in all samples. The following is how the corrected cytokine mean was calculated: $\text{average of each replicate} \times \text{cytokine slope} / 28\text{S slope} \times 28\text{S correction factor}$.

Intestinal Morphology

For intestinal morphology collected duodenum, jejunum, and ileum samples were placed in a 10% middle (PH=7.4) fixed in formalin fixative prepared in paraffin sections. Perform HE staining, observe under an

optical microscope, randomly select 5 discontinuous fields for each slice, and count 3 sets of data for each field. The height of the villi refers to the length from the top of the villi to the base of the villi, and the depth of the crypt refers to the distance from the bottom of the intestinal gland to the base opening between the two villi. Measure villus height (VH), crypt depth (CD), and calculate villus height to crypt depth ratio(VH/CD).

Cecal Microbiota

DNA Extraction

Dewar et al. (2013) as previously described, using a QIAamp Fast DNA stool mini kit (Qiagen), microbial DNA was extracted from cecum samples as per manufacturer's guidelines.

The quality and the quantity of harvested DNA were measured using a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA); additionally, 1 % agarose gel electrophoresis was used to ensure DNA quality. 338 F (5-**ACTCCTACGGGAGGCAGCA**-3) and 806 R (5-**GGACTACHVGGGTWTCTAAT**-3) were used to amplify the V3-V4 hypervariable regions of the bacterial 16S rRNA gene. A thermocycler PCR system (Gene Amp 9700, ABI, USA) was used to conduct PCR and the reactions were performed in triplicates: 20 µL mixture, containing 4 µL of 5°ø FastPfu Buffer, 2 µL of 2.5 mmol/L dNTPs, 0.8 µL of each primer (5 µmol/L), 0.4 µL of FastPfu Polymerase, and 10 ng of template DNA. To purify the extracted PCR products from a 2 % agarose gel, the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) was used, which were then quantified using QuantiFluor-ST (Promega, USA) according to the manufacturer's guidelines.

Pyrosequencing and Bacterial Data Processing

According to Majorbio Bio-Pharm Technology Co. Ltd, Shanghai, China, purified amplicons were pooled and sequenced in equimolar concentrations on an Illumina MiSeq PE300 platform (Illumina, San Diego, USA). The sequencing results were subjected to bioinformatics analysis. Quality-filtered by Trimmomatic and FLASH software with the following criteria were the established raw fastq sequences: (i) bases with a score of less than < 20 were excluded, and at any site with an average quality, the reads were truncatedscore < 20 across a 50-bp sliding window; (ii) Trimmomatic software was used to delete truncated reads that were less than 50 bp in length; (iii) reads of up to 2 mismatched nucleotides and more than 0 barcode mismatches were discarded; (iv) using the FLASH programme, the merged reads were removed; additionally, sequences that overlapped longer than 10 bp were assembled using their overlap sequences; (v) readings that have not been assembled have been excluded. After this, high-quality sequences acquired were paired with samples based on their barcodes, clustering these reads with up to 97 percent similarities in operational taxonomic units using U search (version 7.1). Then, using the Ribosomal Database Project classifier algorithm, the chimaera and singletons were eliminated and allocated to taxa. A BLAST search for taxonomic classification was performed using QIIME at the 70 percent confidence level in the SILVA database (version 1.8.0). The taxonomic composition was investigated at the phylum, family, class, genus, and species levels using Vegan packages in R. Principal

coordinate analysis(PCA) of the complete diversity of microbial communities based on species levels based on Bray–Curtis distance was managed by R; (Xia and Wishart 2011).

Statistical Analysis

Data regarding growth performance, production performance, haematological parameters, carcass, and immunological parameters were analysed using SPSS software (IBM Corp. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY, USA). GraphPad Prism 7.0 software (Chicago, IL, USA). The data are presented as mean $\pm$ SEM. $P \leq 0.05$ was considered to be statistically significant. Prior to variance analysis, the data on broiler mortality were transformed using arc sine (angular) transformation values to achieve a more normal distribution of the data (Zuidhof et al. 1995).

Results

Growth performance

Fermented feed and antibiotics both conveyed growth benefits. During the first 21 days, the body weight of FFH and FFL groups were improved than the NC group, but no significant differences were found in the body weight, FCR, ADG, and ADFI among the four groups ($P > 0.05$; Table 3). No growth performance indices were changed significantly at the (22-42 day) period. The FFL and PC groups have been improved and significantly lower FCR over the NC group but trends towards lower in the FFH group than the NC group from 0-42 ($P < 0.05$). In the whole period (0 to 42 day) there is no significant difference, in any other growth performance indices. Mortality was also not significant for any time period but higher in the FFL group than NC and PC groups while no mortality was found in the FFH group during 0 to 21 day ($P > 0.05$ Table 3), as due to treatment effects, lower mortality was observed during 21 to 42 day and complete period of 0 to 42 day.

Slaughter Performance and Immune organs index

All eviscerated ratios, breast and thigh muscles ratio, the relative rate of liver and fat were evaluated, all eviscerated ratio was higher in the PC group than the FFH group ($P < 0.05$). While on the other hand no differences were found for breast muscle, thigh muscle, liver, and abdominal fat rate parameters ($P > 0.05$ Fig. 1).

Immune organs spleen, bursa, and thymus index were also measured. Thymus weight was higher in the NC group than FFL and FFH group ($P < 0.05$) whereas spleen and bursa were not different between groups (Fig. 1).

Duodenum, jejunum, and ileum morphology

The morphometric traits of the digestive tract of chicken in different groups are shown in Fig. 2. The morphology of the duodenum, jejunum, and ileum was significantly affected by diet supplementation with fermented feed, the intestine of all groups showed normal tissue architecture. The chicken fed

with FFH had greater ($P < 0.05$) villus height for the duodenum than PC, and higher VH/CR for the duodenum and ileum than PC and the FFL group. The FFL chickens had greater ($P < 0.05$) jejunal and ileal villus height than PC and NC groups respectively. The ileal VH/CD was higher ($P < 0.05$) in the FFL group than the other groups. However, jejunum VH/CD was higher ($P < 0.05$) in both FFL and FFH than in the PC group. Nevertheless, the duodenal and ilea crypts were significantly higher in PC and FFL respectively ($P < 0.05$), as compared to the NC group.

Pro-inflammatory cytokine

An increase in the gene expression of pro-inflammatory cytokine IL-1 β and TLR4 was detected in the PC group than the NC group ($P < 0.05$; Fig. 3A, B), whereas IL-10/ β was higher in the FFL group than the NC group, although similar between PC, FFL, and FFH groups, while IL-4/ β , IFN- γ / β , and IL-6/ β were not different between groups.

Analyses of microbiota composition

The PCA analysis was applied to the data to illustrate the variation of the caecal microbiota between the groups. PC1 explained 33.06% of the variance and PC2 explained 26.87% (Fig. 4). The PCA revealed that the species of cecal samples among the groups were separated from the control NC group.

We observed that the dominant phyla in all four groups were *Firmicutes*, *Bacteroides* and *Proteobacteria* (Fig. 5A). The *Bacilli*, *Bacterioda*, *Clostridia*, and *Negativicutes* were the dominant class (Fig. 5B). The dominant family were *Lactobacillae*, *Bacterideceae*, and *Rikenellaceae* (Fig. 5C). Whereas the *Lactobacillus*, *Bacteroides*, and *Alisptes* were the dominant genus (Fig. 5D). The dominant species included *Bacteroidesphelibetes*, *Lactobacillus aviarus*, *Bacteriodes sp millerius* (Fig. 5E). In different groups, no significant differences were found in the abundance of major phylum *Firmicutes* and *Bacteroides*, whereas FFH and PC groups have a lower abundance of *Delsulfobacterota* phylum compared with FFL and NC groups ($P < 0.05$; Fig. 6). The class of *Desulfovibriona* was also decreased in the group treated with FFH and PC over FFL and NC groups ($P < 0.05$; Fig. 6), while the abundance of class *Negativicutes* a gram-negative bacterium that belongs to phylum *Firmicutes* were considerably declined in FFH compared with other groups ($P < 0.05$; Fig. 6). The family having well-known probiotic bacteria the *Lactobacillaceae* were significantly increased in the FFH and FFL fermented feed groups compared with PC and NC groups ($P < 0.05$; Fig. 6). Fermented feed groups FFH and FFL also increased the abundance of lactic acid producing genus *Lactobacillus* ($P < 0.05$; Fig. 6). FFH increased the abundance of the *Lactobacillus aviarus* species followed by the FFL group compared with other groups ($P < 0.05$; Fig. 6). Compared with the control NC group, the *Lactobacillus aviarus* species abundances were reduced in the PC group ($P < 0.05$; Fig. 6).

Discussion

Microbial fermentation has recently been developed as a cost-effective means to increase the nutritional value of broiler feed. The demand for fermented feed has risen as a consequence of the GIT health and

growth performance benefits for the broiler industry's use of the fermenting process (Chiang et al. 2009; Skrede et al. 2003). Many reports have shown that fermentation of feedstuff with *Lactobacillus* can promote growth performance in chicken (Chiang et al. 2009; Skrede et al. 2003), although some studies have also reported that fermentation of feedstuff with *Lactobacillus* does not affect growth performance (Xu et al. 2012). In this study on the 21st day, fermented feeds FFL and FFH groups showed improved weight gain as PC and, at the end of the experiment, the PC and FFL group has a significantly lower FCR. As demonstrated by Skrede et al. (2003) the positive effect of *Lactobacillus* fermentation on weight gain and feed conversion was found on wheat and barley in the diet. Improved feed conversion suggested a positive outcome of fermentation on nutrient digestibility and energy utilization, as confirmed in other studies (Skrede et al. 2001). The development of a well-balanced microflora given by fermented feed in the broiler diet may be responsible for improved growth efficiency. Proper fermented feed supplementation can create a favourable environment in the intestines for the colonisation of beneficial microflora, resulting in improved broilers' growth efficiency (Mohnl 2011).

The enhanced digestion and absorption that is obtained from fermentation is believed to lead to improved bird productivity (Dražbo et al. 2018). There is a powerful connection between increased villus height and the function of intestinal villi (Shamoto and Yamauchi 2000). In our study, the chicken fed with FFH had greater ($P < 0.05$) villus height and higher VH/CR for the duodenum and ileum. The FFL chickens had greater ($P < 0.05$) jejunal and ileal villus height and greater ileal VH/CD ($P < 0.05$). However, jejunum VH/CD was higher ($P < 0.05$) in both FFL and FFH groups. Previous studies have shown that diets with fermented products have a beneficial effect on the structure and function of the small intestine in poultry (Hou et al. 2013; Liu et al. 2008) and as a result enhanced performance parameters (Dražbo et al. 2018; Jazi et al. 2018).

Fermented feed can improve histology by inhibiting excessive inflammation in the GIT, according to previous studies of Missotten et al. (2013), a recent studies link feeding the chicken lactic acid-fermented diets, which are usually higher in LAB bacteria, to increased resistance to infectious disease within those birds. Although the precise immunomodulatory activities of LAB in broilers are unknown, they may cause various subsets of immune cells to produce cytokines, which play important roles in the induction and regulation of immune responses. There is some evidence that some strains of *Lactobacilli* down-regulated TLR4 expression (Bermudez-Brito et al. 2013). IL-10 is generally known to be an anti-inflammatory cytokine, IL-10 production and regulatory T-cells are involved in the immune tolerance to intestinal microbiota (Cong et al. 2002; Kullberg et al. 2002). In our study IL-1 β , TLR4, and IL-6 expressions were decreased and IL10 expressions were increased with fermented feed FFL and FFH. This is in agreement with our finding that dietary fermented soybean meal in animals decreased IL-1 β concentrations and increased IL-10 concentration than their corresponding controls (Zhang et al. 2018a). However, aside from better intestinal immunity, the poultry's immunity is normally increased in addition to a diet containing fermented ingredients, thus resulting in a higher weight of the bird's immune organs. To determine the immune system of animals, the relative weight of lymphoid organs is mostly used (SA et al. 2008). In the present study thymus index of the NC group was significantly higher than FFL and FFH

group while no significant differences were found between different groups in spleen and bursa weight. Similarly, Tang et al. (2012) and Choi et al. (2014) found no significant differences in the relative weights of the spleen, thymus, and bursa of Fabricius in broilers after feeding with fermented feed. The above findings suggest that lymphoid compartments vary in their responses to the inclusion of fermented feed in chicken due to the different roles in the immune system. The bursa of Fabricius and spleen are sites of differentiation of B cells and T cells, respectively (Cooper et al. 1966). Variations in thymus weight could be associated with changes in the lymphoid organs' function. Hence higher thymus index in the NC group might be due to higher exposure of infections or a reduced capacity to maintaining production potential to meet the sanitation challenges (Fasina et al. 2006). It is important to realise that the lowest weight may not necessarily be linked with lower production of lymphoid cells; so this is essential that also consider other parameters of immunity status (Kabir et al. 2004; Makram et al. 2010).

The PCA revealed that the species of cecal samples among the groups were clearly separated from the NC control group. In contrast, despite no obvious differences in the microbiome in the antibiotic-group PC and fermented feed groups, based on PC2, indicating that the species of cecal samples might be influenced by the host feed, change in intestinal absorption, dysbiosis in ceca of the chicken broiler. The gut bacterial community and the abundance of bacterial metabolites are affected by diets, consequently, the gut microbiota composition influences nutrient digestibility (Fan et al. 2015; Fan et al. 2017). FFH and PC groups have a lower abundance of *Delsulfobacterota* phylum and class of *Desulfovibrionia* compared with FFL and NC group ($P < 0.05$; Fig. 6). On the other hand *Desulfobacterota* phylum (formerly *Deltaproteobacteria*) are highly enriched with intestinal inflammation and injury in the gut of infected chickens (Oakley and Kogut 2016; Yitbarek et al. 2018), while the abundance of class *Negativicutes* a gram-negative bacterium belongs to phylum *Firmicutes* which were significantly decreased in FFH group compared with other groups. The members of the *Negativicutes* family have a gram-negative cell wall composition (Sutcliffe 2010). Lower abundance in FFH and PC groups may suggest that fermentation effectively reduced infection and harmful bacteria. Stimulatingly, lactic acid produced by *Lactobacillus* is necessary for antimicrobial activities to suppress the development and growth of virulence factors of Gram-negative bacteria (Zhang et al. 2018b). The mixture of *P. acidilactici* and *L. fermentum* may drop the *Anaerovibrio* by promoting lactic acid production, which filters through the gram-negative bacterial outer membrane (Alakomi et al. 2000). *Lactobacillus* aviarus was significantly increased in the FFH and FFL fermented feed groups compared with PC and NC groups ($P < 0.05$; Fig. 6). *Lactobacillus* sp. is well known for its beneficial effect on humans and animals, so a high level of *Lactobacillus* sp. is associated with chicken health (Coeuret et al. 2004). The previous research has also shown that feeding with *Lactobacillus* strain significantly increases the diversity of *Lactobacilli* in the ileum and cecum of broilers (Chim-anage et al. 2008; Nakphaichit et al. 2011). These changes in cecal microbiota towards positive increase beneficial bacteria in FFL and FFH.

Conclusion And Perspective

This study demonstrated that fermented feed exhibits dose-dependent beneficial influences on gut microbiota and morphology and immune functions birds. Although the growth promoting effect, such as

feed efficiency, of fermented feed was not as good as that of antibiotics, it was significantly better than antibiotics in reducing the chickens’ mortality. However, limited data is available, so future studies must be consistent in their study approaches, as well as maintaining awareness of how feed fermentation ingredients affect the broiler population's microbiota.

Abbreviations

NC:	Negative control basal diet
PC:	Positive control basal diet +antibiotic
FFL:	Basal diet + Fermented feed low dose
FFH:	Basal diet + Fermented feed high dose
FCR:	Feed conversion ratio
ADG:	Average daily gain
ADFI:	Average daily feed intake
BW:	Body weight
IL-1β:	Interleukin 1 beta
IL-6:	Interleukin 6
IL-10:	Interleukin 10
IL-4:	Interleukin 4
IFNγ:	Interferon gamma
TLR4:	Toll-like receptor 4
qRT-PCR:	Quantitative real-time PCR
VH:	Villus height
CD:	Crypt depth
AGPs:	Antibiotic growth promoters

Declarations

Availability of data and materials

The datasets generated and/or analysed during this study are available from the corresponding author on reasonable request.

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Author contributions

Wentong Peng, Mir Zulqarnain Talpur, Gang Shu, and Haijun Zhang contributed to the design and implementation of the research, to the analysis of the results also helped in the elaboration of the project and its execution and financing. Yuxian Zeng, and Peipei Xie Jincheng Li, Songbo Wang, Lina Wang, and Xiaotong Zhu participated in the execution of the experiment and collection of samples and data. Ping Gao and Qingyan Jiang did the laboratory analysis. All authors discussed the results and contributed to the final manuscript.

Ethics declarations

Ethics approval

The animal trial was approved by the Animal Ethics Committee at The South China Agricultural University under approval number: ACE-CAAS-20201211.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing no financial competing interests.

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Tables

Table 1 Test diet formula and nutritional level

Items	Starter diet	Grower diet
Ingredient%	0~21 d	21~42 d
Corn	56.59	59.96
Soybean meal	25.95	20
Cotton meal	4.5	4.42
Wheat meal	4	5
Wheat middlings	2	2
Oil	2.49	4.5
Calcium hydrogen phosphate	1.82	1.58
Mountain flour	1.35	1.27
Salt	0.35	0.35
Lysine	0.35	0.35
Methionine	0.23	0.21
Threonine	0.05	0.04
Premix of trace elements [□]	0.2	0.2
Vitamin premix [□]	0.02	0.02
Choline chloride	0.1	0.1
Total	100	100
Nutrition level [□]		
Metabolizable energy (kcal/kg)	2980	3160
Crude protein (%)	21.95	19.95
Calcium (%)	1	0.9
Available phosphorus (%)	0.45	0.4
Lysine (%)	1.30	1.15
Methionine (%)	0.58	0.54
Methionine+Cystine ^{□%□}	0.94	0.87
Threonine (%)	0.84	0.75
Tryptophan (%)	0.23	0.2

Note: ¹ Premix of trace elements (provides milligrams per kilogram of feed): Cu 8, Zn 75, Fe 80, Mn 100, Se 0.15, I 0.35

¹Vitamin premix (provided per kilogram of feed): Vitamin A 12 500 IU, Vitamin D3 2500 IU; The following are mg: Vitamin E 18.75, Vitamin K3 2.65, Vitamin B12, Vitamin B2 6, Vitamin B12 0.025, Biotin 0.0325, Folic acid 1.25, pantothenic acid 12, niacin 50

¹ the results are calculated values

Table 2 Real-time quantitative RT-PCR probes and primers

Gene	Sequence type-Probe/primer sequence	Accession number
IL-1 β	forward 5'-TTCATTACCGTCCCGTTG-3' reverse 5'-GCTTTTATTTCTCCAGTCACA-3'	☐NM_204524.1☐
IL-6	forward 5'-AAATCCCTCCTCGCCAATCT-3' reverse 5'-CCCTCACGGTCTTCTCCATAAA-3'	☐NM_204628.1☐
IL-10	forward 5'-CGCTGTCACCGCTTCTTCA-3' reverse 5'-TCCCGTTCTCATCCATCTTCTC-3'	☐NM_001004414.2☐
IL-4	forward 5'-GTGCCCACGCTGTGCTTAC -3' reverse 5'-AGGAAACCTCTCCCTGGATGTC-3'	☐NM_001007079.1☐
IFN γ	forward 5'-GCCCTTCCTGTAACCAGATG-3' reverse 5'-ACACGACAGCCAAGTCAACG-3'	☐NM_205149.1☐
TLR4	forward 5'-TTTCCAAGCACCAGATAGCAACA-3' reverse 5'-TTCCAGCACAAGCCCTGAAATTA-3'	☐NM_001030693.1☐

Interleukin 1 beta, Toll-like receptor 4, Interleukin 6, Interleukin 10, Interleukin 4, Interferon gamma.

Table 3 Effect of fermented feed supplementation and antibiotic on growth performance in broiler chicks¹

Items ²	NC	PC	FFL	FFH	P-value
1-21day					
ADG, g	40.53±0.70	41.69±2.43	41.48±2.51	42.87±1.37	0.32
ADFI, g	48.31±2.60	48.42±2.44	48.85±1.88	50.49±1.53	0.38
FCR (g:g)	1.19±0.05	1.16±0.01	1.179±0.03	1.178±0.30	0.62
Mortality ³	0.19±1.19	1.19±1.19	3.33±2.04	0	0.16
21 d BW	904.90±19.94 ^b	931.66±53.69 ^{ab}	971.02±36.42 ^a	972.16±24.75 ^a	0.02
22-42 d					
ADG, g	76.90±15.98	71.01±6.61	77.28±16.94	80.55±9.97	0.71
ADFI, g	140.42±23.92	131.68±13.59	142.60±24.86	148.85±13.33	0.59
FCR (g:g)	1.840±0.090	1.853±0.020	1.863±0.110	1.855±0.089	0.98
Mortality ³	8.44±2.57	4.87±1.72	1.82±1.82	3.33±2.04	0.34
42 d BW	2891.27±376.0	2762.44±209.0	2863.69±435.3	3058.68±203.9	0.55
42 d					
ADG, g	58.04±7.87	56.61±6.06	58.46±8.95	60.93±4.79	0.81
ADFI, g	92.70±11.58	88.64±8.46	93.39±12.61	97.69±6.16	0.57
FCR (g:g)	1.612±0.013 ^b	1.567 ±0.022 ^a	1.590 ±0.050 ^a	1.605 ±0.042 ^{ab}	0.05
Mortality ³	9.63±2.88	6.06±1.57	5.15±3.47	3.33±2.04	0.63

1n = 6 replicates per treatment.

2Negative control (NC), Positive control (Antibiotic) (PC), Fermented feed (low dose) (FFL), Fermented feed (high dose) (FFH).

3Values transformed by arc sine (angular) transformation.

ADG, average daily gain; ADFI, average daily feed intake; FCR (feed: gain = g: g), feed conversion ratio; BW, body weight.

Note☐Values with different superscripts in the same row differ (P < 0.05).

Figures

Figure 1

Effect of antibiotic and fermented feed on eviscerated yield, component parts, meat ratios, and immune organs in broiler chicken. Negative control (NC), Positive control (Antibiotic) (PC), Fermented feed (low dose) (FFL), Fermented feed (high dose) (FFH). Values with different superscripts in the same row differ ($P < 0.05$). Different color bar plots showing different groups. $n = 6$ replicates per treatment.

Figure 2

a. Effect of antibiotic and fermented feed on duodenum, jejunum, and ileum in broiler chicken. Negative control (NC), Positive control (Antibiotic) (PC), Fermented feed (low dose) (FFL), Fermented feed (high dose) (FFH). Values with different superscripts in the same row differ ($P < 0.05$). Different color bar plots showing different groups. $n = 6$ replicates per treatment. b. Effect of antibiotic and fermented feed on small intestine's (duodenum, jejunum, and ileum) morphology in broiler chicken. Negative control (NC),

Positive control (Antibiotic) (PC), Fermented feed (low dose) (FFL), Fermented feed (high dose) (FFH). The gross and microscopic views of different parts of chickens' intestine in treatment groups and control group. The intestine parts duodenum, jejunum, and ileum sections were used. Histology was assessed for intestine sections embedded in paraffin – sections stained with hematoxylin and eosin. n = 6 replicates per treatment.

Figure 3

Effect of antibiotic and fermented feed on the relative gene expression of inflammatory cytokines of broiler chickens. Negative control (NC), Positive control (Antibiotic) (PC), Fermented feed (low dose) (FFL), Fermented feed (high dose) (FFH). Values with different superscripts in the same row differ (P < 0.05). Interleukin 1 beta, Toll-like receptor 4, Interleukin 6, Interleukin 10, Interleukin 4, Interferon gamma. n = 6 replicates per treatment.

PCA on species level

Figure 4

PCA on species level. Negative control (NC), Positive control (Antibiotic) (PC), Fermented feed (low dose) (FFL), Fermented feed (high dose) (FFH).

Figure 5

Effect of antibiotic and fermented feed on the Relative Abundance of broiler chickens. Negative control (NC), Positive control (Antibiotic) (PC), Fermented feed (low dose) (FFL), Fermented feed (high dose) (FFH). The microbiome compositions in cecum at phylum, class, family genus, and species-level composition of the caecal microbiome of chicken. A color-coded bar plot shows the average bacterial phylum, class, family, genus, and species distribution in different treatment groups and control groups. Each bar chart represents the relative abundance of each group. Each color represents a specific bacteria phylum (A) a specific bacteria class (B) a specific bacteria order (C) a specific bacteria family (D) a specific bacteria genus (E) and a specific bacteria species (F)

Figure 6

Effect of antibiotic and fermented feed on the H-test bar plot on phylum, class, order, family, genus, and species level of broiler chickens. Negative control (NC), Positive control (Antibiotic) (PC), Fermented feed (low dose) (FFL), Fermented feed (high dose) (FFH). The bacterial clades showing differences at phylum, class, order, family, genus, and species levels in different treatment groups and control groups. (A) bacterial clades significantly different at the phylum level in different groups ($P < 0.05$ is showing significant difference). (B) bacterial clades significantly different at the class level in different groups ($P < 0.05$ is showing significant difference). (C) bacterial clades significantly different at the order level in different groups ($P < 0.05$ is showing significant difference). (D) bacterial clades significantly different at the family level in different groups ($P < 0.05$ is showing significant difference). (E) bacterial clades significantly different at the genus level in different groups ($P < 0.05$ is showing significant difference). (F) bacterial clades significantly different at the species level in different groups ($P < 0.05$ is showing significant difference).