

Supplement of probiotics improves the meat output and quality of near maturity yellow-feathered broilers

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Abstract

To investigate effects of *Lactobacillus plantarum* and *Bacillus subtilis* as feed additives on the growth performance of near maturity yellow-feathered broilers. A total of 75 100-day-old hens were randomly divided into five groups. 1% of *L.plantarum*(P-group), *B.subtilis* (B-group), a 1:1 mixture of both strains(PB-group), or commercial probiotic production M-group were added into the drinking water at a concentration of 1.5×10^9 cfu/mL respectively. The control group was fed basal diet. Our results showed that *L.plantarum* could increase the intestinal enzyme activity of broilers, while decreasing the plasma MDA content, increasing the ALB and TP content, decreasing the plasma AST content, increasing the concentrations of plasma IgA and CD8 molecules, improving the jejunum structure. Meanwhile, *B.subtilis* increased the concentration of plasma AST and CD8 molecules of broilers. Additionally, *B.subtilis* also increases the plasma GSH-Px content, but not affecting the plasma biochemical indexes. And increases the plasma IgG and GSH-Px contents and the VCR ratio also improved in M-group. Additionally, the methionine content in thoracic muscle was increased in all groups. But only adding probiotics groups increase the relative abundance of broiler intestinal phylum *Synechococcus* and Phylum *Firmicutes* while decreasing the relative abundance of *Enterococcus* spp. Our results showed that adding probiotics could improve the meat output and quality. It might also be necessary to optimize the feeding conditions by selecting specific strains according to different effects of those probiotics, and to evaluate the best viable number of probiotics which survives in broiler guts to balance the cost and the effort.

1 Introduction

Due to the pandemic, the local poultry industry suffered heavy losses. Currently, the Chinese market shown increasing needs of frozen fresh poultry products which pushing out changes of the selection criteria for broilers. Broilers with glossy feather, warm heads and feet as well as eyes, and the animal welfare correlated health status became important criterions for the market. Additionally, for frozen products, the size and luster of the chicken breast and leg muscles are also critical evaluation scales which affect vendor's decision-making process. Currently, the breeding model of local industry is a combination of three stages: 1) chick brooding in cages with adequate room, 2) then raising in cage-free mode till broilers around 90-day-old; 3) finally, they were caged feeding again for no more than one month before sell to reach a uniform slaughter standard. By using the combination of free-moving and cage rearing, the local enterprise can effectively reduce the tension during transportation and minimize the adverse effects of stress.

Probiotics have been used as an alternative to antibiotic growth promoters. Because it effectively solved the issue of developing drug-resistant bacterial strains in the broiler industry, it attracts lots of interest in the field [1]. Advantages of using probiotics include the following aspects: it can competitively inhibit pathogens and prevent bacteriocins producing. It regulates enzymatic activities which could change the metabolism of some carcinogens and toxic substances. It also increases the producing of volatile fatty acids to maintain the energy balance and regulatory functions of surrounding tissues. Additionally, probiotics increase intestinal cell adhesion and mucin production, modulating the activity of intestine-associated lymphoid tissues and improve the health of broilers' immune system. Based on results from previous studies, it is generally accepted that lactic acid bacteria (such as the *Lactobacillus* spp.) and the *Bacillus* spp. are the most commonly used probiotics in poultry farming practices. Abundant evidences shown that *L.plantarum* and *B.subtilis* can improve growth performance, enhance immune responses, and modulate the activities of antioxidant enzymes and digestive enzymes in broilers. [2-6]

In this study, 100-day-old broilers feed with diet adding different types of probiotics daily for one month. We then analyzed the effects of probiotics on amino acid content of breast muscle, intestinal digestive enzyme activity, plasma antioxidant index, plasma biochemical index, plasma immune index, jejunum morphology and the change of broiler intestinal flora. Generally, this study tends to provide data to investigate whether adding probiotics could improve broiler growth performance, promote fattening, increase intermuscular fat content, and thus increase the flavor and freshness of chicken meat and achieve uniform slaughter standards.

2 Materials And Methods

2.1 Materials

Three sets of probiotics: *L.plantarum* (1.5×10^9 CFU/ml), *B.subtilis* (1.5×10^9 CFU/ml), compound probiotics (*L.plantarum*: *B.subtilis* were mixed in 1:1 ratio with a final concentration of 1.5×10^9 CFU/ml) were prepared and kept by Guangxi Veterinary Research Institute. The commercial compound probiotic reagent was purchased from a local biological company (Heilongjiang Knod Biotechnology Co., Ltd.). The test rations were prepared according to the Chicken Feeding Standard (Chinese standard: NY/T 33-2004) combined with the growth cycle and breed characteristics of the yellow-feather broilers. The composition and nutritional levels of those diets are shown in Table 1.

2.2 Experimental design and the broiler feeding protocol

A total number of 75 100-day-old healthy yellow-feather broilers (hens) with an average body weight of (626.71 ± 1.95) g were randomly divided into five groups (15 for each), and the average initial body weight among groups were not significantly different. The diet of each group was designed as following: 1) normal control group (C) was fed the normal diet; 2) *L.plantarum* group (P) was fed by the normal diet, while adding *L.plantarum* liquid into the drinking water at a concentration of 1.5×10^9 cfu/mL; 3) *B.subtilis* group (B) was fed the normal diet, while *B.subtilis* liquid at a concentration of 1.5×10^9 cfu/mL was added to the drinking water. 4) Probiotic complex group (PB) was fed the basal diet, while probiotic complex (1:1 ratio of *L.plantarum*: *B.subtilis*, final concentration of 1.5×10^9 cfu/mL) was added to the drinking water; 5) The commercial probiotic complex group (M) was feed by normal diet with 1% of commercial enzyme preparation. The probiotic live bacterial solution was changed every 12 hours during the experiment.

The test was conducted at the experimental base of Guangxi Veterinary Research Institute, and the test chickens were purchased from one of the top 10 yellow-feather broiler breeding groups in China. The chickens were kept in cages with natural light, with enough living space, free to feed and drink, while their daily feeding and health conditions were monitored and recorded consecutively for 30 days. On day 15 and day 30 of the experiment, three samples from each group for sampling.

2.3 Measurements of multiple broiler indexes

2.3.1 The quality analysis of poultry meat

The breast muscles of chickens were taken after slaughtering and sent to the Analysis and Testing Research Center of Guangxi Zhuang Autonomous Region. The amino acid content of the breast muscle was determined.

2.3.2 Determination of Immune organ indexes

Broiler chickens were extracted and weighed alive, slaughtered, then remove their spleen and thymus, isolating fat tissue and weighed. The thymus index and the spleen index were calculated and recorded separately. Thymus index refers to the weight of the organ (mg) per 10 g of body weight. It was calculated by the formular: $\text{Thymus index} = [\text{thymus weight (g)} \times 10^3] / [\text{live chicken weight (g)} / 10] = [\text{thymus weight (g)} / \text{live chicken weight (g)}] \times 10^4$. Spleen index refers to the weight of this organ (mg) per 10 g of body weight. It was calculated by the formular: $\text{Spleen index} = [\text{spleen weight (g)} \times 10^3] / [\text{live chicken weight (g)} / 10] = [\text{spleen weight (g)} / \text{live chicken weight (g)}] \times 10^4$.

2.3.3 Intestinal enzyme activity assay

The jejunum and cecum segments were collected and then briefly raised with pre-cooled saline (4°C). All tissue were drained and 0.1g of each intestinal segment was weighed. After adding 0.9mL of saline in each sample, tissue was homogenized and centrifuged at 2500 r/min for 10 minutes. The supernatant was extracted and stored at 4°C before using. Enzyme activities of each intestinal segment were determined according to the instructions of Elisa kits for testing the amylase, lipase and chymotrypsin. These Elisa kits were purchased from Jiangsu Jingmei Technology Co., Ltd.

2.3.4 Determination of plasma antioxidant indicators

For each sample, 5mL blood samples were collected by non-anticoagulated blood collection tubes and then centrifuged at 3000 r/min for 20min to isolate the plasma. Total antioxidant capacity (T-AOC), Superoxide dismutase (SOD), Glutathione peroxidase (GSH-Px) and Malondialdehyde (MDA) were tested from each of plasma samples. All testing operations and the following calculation were performed by the instructions of T-AOC, SOD, GSH-Px and MDA assay kits. These kits were purchased from Nanjing Jiancheng Institute of Biological Engineering Ltd.

2.3.5 Measurement of plasma biochemical indicators

For each sample, 2mL blood was collected by anticoagulated blood collection tube and collecting supernatant (centrifuged at 3000r/min for 20min). Plasma Total protein (TP), Albumin (ALB), Triglycerides (TG), Total cholesterol (TC), Phenylalanine transferase (ALT) and Aspartate transferase (AST) were then tested. The operation and calculation procedures were performed in strict accordance with the relevant kit instructions. TP, ALB, TG and TC kits were purchased from Nanjing Jiancheng Institute of Biological Engineering Ltd. The ALT and AST ELISA kits were purchased from Jiangsu Jingmei Technology Co., Ltd. The experimental results were determined by Spark™ 10M multimode microplate reader (Tecan Lifesciences).

2.3.6 Measurement of plasma immunological indicators

The ELISA method was used for the determination of IgG, IgA, IgM, CD4 and CD8. The operation and calculation process were carried out in strict accordance with the instructions of the kit. The experimental results were determined by Spark™ 10M multimode microplate reader (Tecan Lifesciences).

2.3.7 Morphological observations of the jejunal intestine

On day 15 and day 30 of the experiment, three samples from each group were sacrificed after overnight fasting. The jejunal segments were selected from the same locations, gently rinsed with saline to remove their intestinal contents, and fixed in 4% paraformaldehyde. The fixed jejunum samples were embedded in paraffin blocks, then 8 µm tissue sections were prepared. Slices were stained with eosin hematoxylin and their morphological structures of the intestine were observed under a light microscopy. The villi height and the intestinal gland depth of the jejunum were measured and the villi height/ intestinal gland depth ratio (VCR) was calculated for each sample. The villi height (µm) was measured from the tip of the villi to the junction of the villi crypt. The intestinal gland depth was measured as the depth of the adjacent intestinal gland villi invagination.

2.3.8 The collection and high-throughput detection of intestinal contents

Three testing samples from each group were randomly selected to sacrificed, and 2 mL of intestinal contents were collected aseptically and stored at -80°C till further tests. Total DNA was extracted from the intestinal samples using the total DNA Purification Kit. The total DNA extraction were further isolated by agarose gel electrophoresis, collected and purified, then DNA fragments with different molecular weights were quantified by ultra-micro spectrophotometer. The target sequences (such as microbial ribosomal RNA, which can reflect the composition and diversity of the flora) were used as target sites and the corresponding primers were designed according to the conserved regions in these sequences. Then sequencing universal junctions and sample-specific barcode sequences were added to the universal primers, following by PCR amplification to get the variable region (V3+V4) of the bacteria 16srDNA gene or specific gene fragments:

338F ACTCCTACGGGAGGCAGCAG; 806R GGACTACHVGGGTWTCTAATPCR. The amplification products were further isolated by 2% agarose gel electrophoresis and the specific band with the correct size of target fragment were collected and purified. For further analysis, we confirmed that the concentration of all qualified DNA fragments was above 2nM (quantified by using Qubit Fluorometric Quantification, Thermo-fisher). The MiSeq benchtop sequencer (Illumina) was then used for the double-end sequencing. Qualified libraries were diluted in a gradient concentration according to the required sequencing volume, and denatured to single strand by NaOH before sequencing. Qualified sequencing results were normalized by the volume of each sample for further comparisons. According to at least 20bp of the end overlapping regions, the two consecutive DNA fragments were stitching together. To get high quality sequencing results, we excluded the following unqualified results: 1) sequences containing 'N' in the end; 2) Sequencing results with quality value lower than 20 at either terminal sequence; 3) and results less than 200bp after stitching. Then comparing the filtered results with the database to remove the chimeric sequences to get valid data. All valid data from our samples were then clustered using the UPARSE-OTU algorithm(https://drive5.com/usearch/manual/uparseotu_algo.html). These sequences were then clustered into operational taxonomic units (OTU) with 97% OTU threshold, and the OTU sequences were annotated with species using the Mothur method (MiSeq SOP) and the SILVA rRNA database (SILVA132, <https://www.arb-silva.de/>) for species annotation analysis. The taxonomic information was obtained at the taxonomic level of kingdom, phylum, class, order, family, genus, and species, and the community composition of each sample was calculated.

2Statistical Analysis

The experimental data were statistical analyzed using SPSS 25.0. A significant difference was considered as $P \leq 0.05$, a trend of significant difference was considered as $0.05 < P < 0.10$, and all results include their mean+/- standard deviations (SD value).

3 Results

3.1 Adding probiotics to the diets affect contents of amino acids in broiler breast muscle

As shown in Table 1, a total of 16 amino acids were detected from the breast muscle collecting from these 130-day-old broilers. Among all, the glutamic acid has the highest content while the content of methionine is the lowest. The data also shown that the methionine content increases 0.25%, 0.28%, 0.06% and 0.1% separately in P, B, M and BP treatment groups when comparing to group C. Table 2

3.2 Adding probiotic into diets did not show significant changes of immune organ activities among broiler chickens

For indicators which reflecting the activity of immune organs, no significant difference was observed among groups at the 15th and 30th day of our experiment. Table 3

3.3 Adding some probiotics to the diet affects intestinal enzyme activities in broiler chickens

On day 15th, cecum lipase activity in group M was significantly higher than samples from group PB, but not significantly different from group C. The jejunal lipase activity in group PB was significantly higher than group C and group P. All other testing groups show an increase comparing to controls (group C), but the difference was not statistically significant. The cecal chymotrypsin activity of group M was significantly higher than group PB, whereas the result from group M was not significantly different from that of group C. There was no significant difference in jejunal chymotrypsin activity among all groups. The cecal amylase activity of group M was significantly higher than that of other probiotic groups and group C; jejunal amylase activity of group M was significantly higher than controls (group C). Table 4

On the 30th day of the test, there was no significant difference in jejunal lipase, amylase and chymotrypsin activities among groups. The cecum lipase activity was significantly higher in group P than in group M. The cecum amylase activity was significantly higher in group P and C when comparing with group PB. The cecum chymotrypsin activity was significantly higher in the enzyme supplemented groups than in other testing groups. Table 4

3.4 Probiotics affect the amount of plasma antioxidants of testing broilers

On day 15th of the test, MDA levels were significantly higher in group C comparing to other testing groups, but there was no significant difference in SOD and GSH-Px levels among groups on day 15. Interestingly, the T-AOC level were significantly higher in group P than in group C at this time point. Table 5

On the 30-day of the experiment, the plasma MDA content of group C was significantly higher than groups of B PB and group M, but not significantly different from group P. The plasma SOD content of group P was significantly higher than that of group PB, and the plasma GSH-Px of group PB and group M was significantly higher than groups C, P and B. There was no significant difference in the plasma T-AOC content among groups. Table 5

3.5 Probiotics lead plasma biochemical changes among testing broilers

At 15th day of the trial, the ALB levels were higher in all groups than in group C, but none of them were significantly different. There were also no significant differences between TP. The TG levels in group P were significantly higher than in groups B, M and C. There were no significant differences in TC, ALT or daily AST level changes among groups. Table 6

At day 30 of the test, the ALB levels in all groups were higher than those in group C, but there were no significant differences. No significant differences between TP and TG among all groups. The TC levels in all groups were significantly higher than those in group C. There were no significant differences in ALT levels among groups. The AST levels in group P were significantly higher than those in groups C and group M. Table 6

3.6 Probiotics affects the level of plasma immunoglobulins among testing broilers

On the 15th day of the test, there was no significant difference in plasma IgM levels among groups. Plasma IgA levels in groups B and P were significantly higher than those in groups C, M and PB. The plasma IgG levels in group M were significantly higher than those in groups C and B. While CD4 levels in groups B and PB were significantly higher than those in group M. The CD8 levels in group P were significantly higher than those in groups C and the composite probiotic group. Table 7

At day30 of the trial, there were no significant differences in IgM, IgA, IgG or CD4 levels among groups, but the levels in group C were lower than those in the other test groups. The CD8 level in the plasma of groups B and P were significantly higher than those in groups M, PB and C. Table 7

3.7 Probiotics lead intestinal morphological changes among jejunum samples from testing broilers

On 15th day of the test, the average length of the Intestinal villi in groups P and B was significantly higher than those in controls (group C). The average depth of intestinal glands in group C was significantly higher than that in other testing groups. The average depth of intestinal gland in group B was the lowest. The ratio of the small intestinal VCR of Group B, Group M and Group PB were significantly higher than Group C. Group C had the lowest VCR value and Group B had the highest one. Table 8

On day 30 of the trial, the chorionic villus length was significantly higher in group P than in groups M and PB, and the average depth of the intestinal gland was lowest in group M and significantly lower than in groups C, P and B. The VCR was significantly higher in group M than in all other groups. Table 8

3.6 Probiotics affect the intestinal flora among testing broilers

On 15th day of the experiment, mainly the *phylum Bacteroidota* and *Firmicutes* are dominated populations. In group M, the relative abundance of *Bacteroidota* reached 71.04%, which is the highest among groups. Compared with controls (group C), the relative abundance of *Bacteroidota* in groups B, M and BP were increased by 7.66%, 11.93% and 0.24%, respectively; while in group P it decreased by 2.28%. At the genus level, *Bacteroides* and *Ruminococcus* were dominant populations in group B; *Bacteroides* and *Rikenellaceae* were dominant populations in groups P, M, PB and C. The relative abundance of *Bacteroides* was 17.56%, 9.46%, 2.87%, and 1.29% higher in groups B, M, P, and PB, respectively, compared with controls (group C). The relative abundance of *Ruminococcus* was 3.4%, 3.46%, 2.16%, and 2.19% higher in group B than in groups M, P, PB, and C, respectively. The relative abundance of *Clostridia* in group P was 2.46%, 2.71%, 3.68%, and 3.15% higher than that in the B, M, PB, and C groups, respectively. (Figure 1)

On the 30-day of the experiment, the absolute dominant populations among all test groups were the phylum *Bacteroidota* and *Firmicutes*. Comparing with controls (group C), the relative abundance of *Bacteroidota* increased by 8.93%, 2.68%, 4.54% and 6.36% in groups B, M, P and PB, respectively. The relative abundance of *Firmicutes* increased by 0.79%, 3.53%, 1.11% and 3%, respectively. The relative abundance of *Proteobacteria* decreased by 1.74%, 3.74% and 3.74% in groups B, M, P and PB, respectively. The relative abundance of *Proteobacteria* in the B, M, P and PB groups decreased by 1.74%, 3.09%, 4.01% and 3.5% respectively. At the genus level, the absolute dominant population among all experimental groups was *Bacteroides*. Comparing with controls (group C), the relative abundance of *Bacteroides* in groups B, P and PB increased by 19.79%, 4.16% and 13.11%, respectively. The relative abundance of *Bacteroides* in group M decreased by 6.8%. The relative abundance of *Ruminococcus spp.* in all experimental groups was higher than that in group C. Comparing with group C, the relative abundance of *Enterococcus (Enterococcus spp.)* decreased by 0.36%, 0.21%, 0.22% and 0.14% in groups B, M, P and PB, respectively. (Figure 1)

4 Discussion

4.1 Adding probiotic to diets affect amino acid contents in broiler breast muscle

As the basic substances constituting proteins, the abundance of amino acids is one of the criteria in evaluating the nutrition level of poultry products. This study analyzed the breast muscle of yellow-feathered broiler and found only methionine level significantly changes but no significant differences among other types of amino acids. Particularly, the methionine content of broilers which feed with *L.plantarum*, *B.subtilis*, commercial complex probiotic preparations and complex probiotics in the diet were 1.69, 1.77, 1.17 and 1.27 times higher than that of the control. Methionine is an essential amino acid for protein synthesis and also a precursor of S-adenosylmethionine (SAM). In transmethylation, methionine is the sole methyl supplier for various acceptors, including nucleic acids, proteins, CpG islands in DNA, and biogenic amines [7]. In poultry industry, most feeds are made from corn and soybeans, and the first limiting amino acid in a typical corn-soybean diet is methionine; therefore, methionine has an important role in broiler nutrition. Methionine deficiency impairs poultry growth [8], increases abdominal fat [9], and causes alterations in immune organs [10]. Fagundes et al [11] found that feeding methionine-deficient diets (0.28%) caused reduced growth performance and feed efficiency in 1- to 22-day-old Cobb broiler males compared to controls (0.49% methionine). Therefore, it was hypothesized that adding probiotics to the diet would improve the quality of the broiler by increasing the methionine content.

4.2 Adding probiotic to diets improves the immunoglobulin levels but not affect the weight of immune organs among testing broilers

The weight of thymus, spleen, and bursa can be used to evaluate the immune status of broilers, particularly chicks. The increase in the absolute and relative mass of organs indicates the enhancement of cellular immunity and humoral function of the organism [12]. It was demonstrated that adding probiotics to the diet helped to enhance the immune function of broilers compared to the control groups [13]. In the present experimental study, there was no significant changes in either thymus or spleen index, both of which reflexing immune organ activities. And there was no trend of weight gain were found in our data. This is consistent with the results of Tang et al [14], where the addition of probiotics had no effect on the immune organ index of broiler chickens at the near-mature stage, and it is speculated that since probiotics mainly affect the early development of immune organs, it is beneficial to the immune organ development and increase the maturation rate of immune organs. However, the immune organs of adult broilers are already matured and shown less sensitive to external stimuli. Therefore, the weight changes on the mature immune organs of broilers at 100 days of age in this experiment was not significant.

4.3 Adding specific strains of probiotics to the diet support intestinal enzyme activities in broiler chickens

The intestinal enzyme activity plays an important role in the digestion and absorption of nutrients, especially during the development. The study of digestive enzymes has been an important part of the research on chicken digestive physiology. Wang et al [15] reported that the addition of *B.subtilis* to the diet could increase the Intestinal lipase activity of broilers significantly. Our data shown that adding *B.subtilis* to the diet for only 15 days could significantly increase the activity of broiler jejunum lipase, which was consistent with previous studies. Similarly, adding of commercial complex probiotic preparations could also significantly increase the activity of broiler jejunum amylase. This study also found that adding commercial complex probiotic preparation into the diet could significantly increase both the cecum lipase and amylase activities in broilers.

On the 30-day of the experiment, there were no significant differences in jejunal lipase, amylase, and chymotrypsin activities among groups. Whereas, adding *L.plantarum* to the diet significantly increased the cecum lipase activity in broilers. Similarly, the addition of commercial complex probiotic preparation also significantly increased the chymotrypsin activity of broiler cecum. This indicates that adding probiotics to the diet effectively increases intestinal enzyme activities therefore improving nutrient uptake by broilers.

4.4 Adding probiotics to the diet enhance the plasma antioxidant-levels among testing broilers

Antioxidant status is highly correlated with the immune system and have been considered as an important indicator of immune function. Antioxidant enzyme activities and oxidation product concentrations are important indicators to evaluate the oxidative status of animals [16].

The levels of MDA, SOD, GSH-Px and T-AOC are all important indicators which correlated to antioxidant capacities. Excess free radicals in the organism can damage proteins, nucleic acids and other biomolecules, which are harmful to the organism. Antioxidant enzymes act by eliminating reactive oxygen species such as hydrogen peroxide, peroxides and superoxide radicals, terminating free radical chain reactions and thus enhancing antioxidant capacities [17]. MDA is a product of lipid peroxidation. The MDA can interact with structural components of biological membranes therefore increasing membrane fragility, improving structural modifications and inducing immune system dysfunction. An increasing number of studies have shown that probiotics can act as a natural antioxidant to protect the cell from oxidative damage. Previously studies also reported that *Lactobacillus* can influence the activity and content of antioxidant enzymes in the body and reduce intestinal oxidative stress damage [18-19].

In this study, the addition of probiotics significantly reduced plasma MDA levels in broilers on 15th day of the trial. On day-30, the addition of *B.subtilis*, complex probiotics, and commercial complex probiotic preparations to the diet significantly reduced plasma MDA levels. This data is consistent with the results of Yu et al [20]. They reported that the use of probiotics alone significantly reduced MDA concentrations in pullets. Whereas adding the *L.plantarum* in this experiment did not reproduce the same effect, presumably due to inter-strain specificity. Antioxidant enzymes such as SOD could prevent oxidative stress, improving poultry quality and avoiding immunosuppression in broilers. In the present study, the addition of commercial complex probiotics and complex probiotics significantly increased the plasma GSH-Px levels, which is significant different from the P and B groups. This may be due to the reduction of *L.plantarum* and *B.subtilis* content in the complex probiotic relative to the addition of probiotics alone, therefore not achieving the effect produced by the use of probiotics alone; there is also the possibility that two probiotics (*L.plantarum*, *B.subtilis*) antagonize each other, thus reducing the effects. Therefore, the subsequent application of probiotics in production needs to be optimized according to the specific strains, age, type of probiotics and live bacteria content of the broiler. Meanwhile, the mechanism of antioxidant effect is complex, and the mechanism of probiotics on the improvement of antioxidant function needs to be further investigated. In general, the addition of probiotics can effectively improve the antioxidant capacity of broiler chickens, and the effect varies depending on the specificity of the strain and the number of live bacteria, but it has a positive effect on the antioxidant activity.

4.5 Positive effects were found on plasma antioxidants of broilers when adding probiotics to the diet

Plasma biochemical indicators could reflect levels of specific substances, indicating changes of the growth status of the organism therefore is useful for the feeding management of the livestock. Plasma ALB and TP levels are the main indicators to evaluating nitrogen metabolic levels of the animal. It could reflect the level of protein absorptions, metabolism of the organism, and albumin levels are positively correlated with the growth rate of the animal [21]. In the present study, the addition of commercial complex probiotic preparations, *B.subtilis*, *L.plantarum* and complex probiotics to the diets increased the plasma ALP content compared to the control group on the 15th day and the 30 days of the test. It suggests that the intake of these probiotics could enhance protein digestion in broilers. Plasma TG and TC levels reflect the strength of fat metabolisms. Additionally, the adipose tissue development and the fat deposition also depend on plasma TG levels. In this experiment, adding *L.plantarum* to the diet significantly increased the plasma TG content on 15th day of the experiment; on day-30, the plasma TC content was significantly increased in all test groups, indicating that *L.plantarum* and *B.subtilis* enhanced the fat metabolism of broilers to some extent and improving the fat deposition of the organism. Changes in AST and ALT activities are the also specific indicators which could be used to determine the hepatocytes activity of the organism and also specific indicators of hepatocyte damages [22]. When hepatocytes are damaged, cell membrane permeability increasing, therefore AST and ALT releasing from the cytoplasm of hepatocytes. Fathi [23] showed that feeding diet with Lactobacillus cultures to chickens significantly decreased ALT and AST activity. In this study, adding either *L.plantarum* or commercial complex probiotic preparations to the diet for 30 days could significantly reduce plasma AST levels. It indicates that the liver function of broilers was normal and the utilization of amino acids by the organism was highly efficient.

4.6 Adding probiotics to the diet enhances the level of plasma immunoglobulins among broilers

IgG, IgM, and IgA are immunoglobulins that functionalized as antibacterial, antiviral, and antitoxin elements which produced by bone marrow-derived B lymphocytes. The immune capacity of the body can be reflected by the level of these immunoglobulins in plasma. Therefore, testing their levels in the plasma reflecting changes of relevant immune functions. Previous studies shown that probiotics can inhibit tumor growth by enhancing systemic antibody levels [24] and by activating innate immunity [25-26]. Systemic immunomodulatory effects are considered to be an important mechanism of probiotic function, and supplementation with probiotics has been reported to recruit immune cells therefore activate immune or inflammatory responses by altering the synthesis of chemokines and cytokines [27].

Wang Jiali et al. reported that by adding yeast and lactic acid bacteria to the basal diet, they significantly increased the IgG level of 21-day-old broilers and the IgA level of 42-day-old broilers. Our results shown that adding *B.subtilis* or *L.plantarum* to the diet significantly increased the plasma IgA level of 115-day-old broilers, and the addition of commercial complex probiotic preparations to the diet could also significantly increased the plasma IgG level of broilers. It has also been shown that the addition of *L.plantarum* to the diet can effectively activate chicken mucosal immune functions by increasing the levels of IgA and IgG [28]. In this study, although data shown no significant differences among IgM, IgA, IgG and CD4 levels at 30 days of the trial, these immunoglobulin levels were higher in all test groups than in control (group C). This suggests that probiotics act as a stimulator of the immune system, but the stimulating effect differs due to the strains. However, the detailed molecular mechanism still need to be fully elucidated.

Adaptive immunity can be subdivided into two main categories: 1)the cell-mediated immunity which targeting intracellular pathogens and 2)the humoral immunity which targeting extracellular pathogens [29]. The cellular immunity is mainly mediated by lymphocytes, meanwhile CD4 and CD8 are important lymphocyte subtypes. They function as "co-receptors" of T cells to against antigens. In the present study, adding of either *L.plantarum* or *B.subtilis* to the diet significantly increased CD8 molecule levels in the plasma on 130-day-old broilers. Thus, the addition of probiotics not only supporting humoral immunity, but also has a good performance in improving cellular immunity.

4.7 Adding probiotics to the diet leads positive intestinal morphological changes among jejunum samples from testing broilers

Intestinal morphology, including duodenal, jejunum, and ileal villi height, intestinal gland depth, and the ratio of villi height to intestinal gland depth, can reflect the intestinal health status of broilers. The increase in the height of small intestinal villi helps to improve the digestion and absorption capacity of the animal organism, while the strong and regular oscillation of intestinal villi can also prevent the colonization of harmful bacteria in the intestine and stabilize the balance of the microbiota [30]. In normal conditions, undifferentiated stem cells in the intestinal gland could also developing to the epithelial cells of the villi. And the number of intestinal mucosal epithelial cells is dynamically replaced due to the continuous shedding of epithelial cells at the tip of the villi, following by the replacement of lately differentiated cells which proliferated from the intestinal gland [31].

An increasing of villi height and the villi height to intestinal gland depth ratio are directly correlated to the increase of the epithelial turnover ratio [32]. Longer villi are associated with the activation of cell mitosis [33]. In the present study, the addition of *B.subtilis* to the diet significantly increased the jejunal villus length in broilers after 15 days of probiotic-diet treatment, while significantly decreasing the jejunal intestinal gland depth. The ratio of jejunal villus height to intestinal gland depth was significantly increased in groups B, M and PB. By adding probiotics and commercial complex probiotic preparations to the diet, it significantly decreased the jejunal intestinal gland depth. This result is consistent with the findings of Awad et al. They reported that adding probiotic products to broiler diets increased the villus height and the ratio of villus height to intestinal gland depth in the duodenum and ileum [34]. By adding a commercial complex probiotic preparation to the normal diet for 30 days, we significantly reduced jejunal intestinal gland depth while increasing the VCR ratio. This finding is consistent with the findings of Kim et al [35] who found that the addition of a complex microbial probiotic preparation to broiler diets increased the VCR value at 35 days of age.

4.7 Adding probiotics to the diet improves the intestinal flora populations among testing broilers

The gut is the natural interface between the intestinal microbiota and the host. The number of bacteria could be far more than the number of cells in the host, possessing millions of diverse genes therefore forming the gut microbiota. By expressing these unique of genes, gut microbiota could initiate many enzymatic reactions that cannot be carried out by the host itself. Thus, the gut microbiota influences many aspects of gut development and strongly contribute to the metabolic of the host. [36] Probiotics are current used to treat gut microbial dysbiosis, restoring regional microbial diversity, and altering disturbed gut microbiota [37, 38]. It is generally accepted that probiotics can modulate the composition of the normal intestinal flora, thereby improving feed conversion ratio and ultimately nutrient digestion by improving absorption through the breakdown of large molecules of substrates in the diet [39]. Bacteroidetes has some probiotic roles, including in helping the host to break down polysaccharides to improve nutrient utilization, promoting immune system development, enhancing host immunity, and maintaining intestinal microecological homeostasis [40]. Kaoutari et.al [41]. reported that intestinal *Bacillus* spp. and *Bacillus* spp. are both strongly contribute to the distribution and abundance of carbohydrate-active enzymes (CAZyme) in human intestinal microorganisms with an improvement of digestion function [42]. In the present study, the addition of probiotics to the diet could increase the abundance of broiler intestinal *Bacillus* spp. and *Bacillus* spp. This is consistent with the finding of Yingchao Song [42] that higher abundance of mesophilic *Bacillus* phylum and *Bacillota* was responsible for high feed conversion efficiency in yellow-feather broilers (also named as Sanhuang chickens). It is suggested that the *Bacillota* and the *Bacteroidota* play a major role in influencing feed efficiency. The *Pseudomonadota* is a majority population of bacteria that includes many pathogenic bacteria such as *Escherichia coli*, *Escherichia coli*, *Salmonella* etc. Johnson et al [43] using data mining found that the abundance of *Clostridium* spp, *Enterococcus* spp and some unidentified genera of *Enterobacteriaceae* were negatively correlated with the productive performance of broilers because of pathogenic bacterial species such as *Clostridium perfringens*, *Enterococcus cecorum*. and avian pathogenic *Escherichia coli*. The relative abundance of *Pseudomonadota* and *Enterococcus* spp. in broiler chickens in the probiotic supplementation group showed a decreasing trend, which was consistent with the results of Johnson's previously study. *Ruminococcus* are an important population in the rumen microbial environment, and they allow the host to efficiently degrade and utilize fiber-containing plant material as nutrients. In the present study, the addition of probiotics to the diet increased the relative abundance of *Ruminococcus*. in the broiler gut. It was suggested to improve the growth performance of broilers. Understanding the bacterial microbiota colonizing the chicken gut can help developing the new generation of probiotic feeding products which can support gut health, reducing the need for using antibiotics, and improve chicken welfare in the poultry industry.

5 Conclusion

The gradual reduction of live poultry markets makes it important to standardize the size and improve the poultry quality of broilers. In this study, 100-day-old broiler chickens were used to study the best strategy to improve broilers growth performances and to meet the high-quality standards. In this study, by adding of either *L.plantarum* or *B.subtilis*, we improved the growth performance and improving the health of near-mature broilers. Diet with these probiotic strains shown multiple benefits, including increasing methionine content, enhancing digestive enzyme activities and antioxidant capacities, altered plasma immunoglobulin levels and altered jejunal morphology. According to our results, different probiotics contributed to different degrees in all indices of near-mature-broilers, and the selection of particular probiotic strains according to their unique effects, as well as ensuring the viability of probiotics is essential in poultry industry. Additionally, it is also interesting to identify weather different combinations of probiotics strains have agonistic or antagonistic effects, which could be a fruitful direction for further research.

Declarations

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

Hao Peng: Conceptualization, methodology, investigation, writing—original draft preparation, funding acquisition **Yangyan Yin:** methodology, software, formal analysis, data curation, writing—original draft preparation **Zhe Pei:** writing—review and editing, investigation **Tianchao Wei:** methodology, writing—original draft preparation, resources **Jun Li:** methodology, investigation **Xiaoning Li:** software writing—review and editing **Changting Li:** software **Huili Bai:** software **Chunxia Ma:** writing—review and editing **Yu Gong:** writing—review and editing **Yuyin Liao:** Conceptualization, formal analysis, resources, supervision, project administration, funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

All experimental protocols were approved by Guangxi Department of Science and Technology. All animal procedures were performed in accordance with the protocols approved by the Institutional Animal Experimental Ethical Inspection Form of Guangxi Veterinary Research Institute, Nanning, China(8/2014/JU).The study was carried out in compliance with the ARRIVE guidelines.

Conflict of Interest

This manuscript has not been simultaneously submitted for publication in another journal and been approved by all co-authors. The authors declare that they do not have any conflict of interest.

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Tables

Table 1. Diet composition and nutritional content

composition	%	Nutritional composition index	Unit	Content
Corn	64.99	Metabolic energy	K cal/kg	17.00
Soybean meal	25.00	Crude protein	%	18.33
Wheat bran	5.00	Methionine	%	0.47
Fish Meal	1.00	Lysine	%	1.07
Stone meal	1.20	Methionine+Lysine	%	0.79
Salt	0.30	Calcium	%	0.89
Calcium bicarbonate	1.20	Total phosphorus	%	0.63
Lysine	0.13	Effective phosphorus	%	0.39
Methionine	0.18			
Premix	1.00			
Total	100			

*Each kg of premix contains: vitamin E 15 050 mg, vitamin A 2 636 900 IU, vitamin D 3890 200 IU, vitamin B1 622.0 mg, vitamin B2 2 032.3 mg, vitamin B6 1 138.5 mg, vitamin B12 6.0 mg, vitamin K3 851.9 mg, pantothenic acid 3 245.4 mg, niacin 12 170.3 mg, folic acid 12 170.3 mg,

biotin 60.8 mg, choline chloride 202.8 mg, copper 4 259.6 mg, iron 30 425.9 mg, zinc 30 425.9 mg, manganese 32 444.3 mg, selenium 150.1 mg, iodine 344.8 mg.

Table 2. Effect of probiotics on amino acid content in broiler chicken breasts (130 days of age, 30-days of treatment).

Amino acids(g/100g)	P	B	M	PB	C
Aspartic acid	2.14	2.05	2.19	2.12	2.19
Threonine	1.04	1	1.06	1.02	1.06
Serine	0.91	0.82	0.86	0.84	0.86
Glutamic acid	3.34	3.22	3.42	3.25	3.5
Glycine	1.09	0.93	1	1	1.02
Alanine	1.36	1.31	1.38	1.36	1.39
Valine	1.21	1.15	1.26	1.22	1.24
Methionine	0.61	0.64	0.42	0.46	0.36
Isoleucine	1.12	1.11	1.2	1.16	1.19
Leucine	1.93	1.88	1.98	1.9	1.98
Tyrosine	0.81	0.74	0.74	0.73	0.74
Phenylalanine	0.95	0.87	0.96	0.96	0.96
Histidine	0.76	0.7	0.94	0.89	0.7
Lysine	2.03	2.04	2.14	2.07	2.2
Arginine	1.54	1.48	1.51	1.47	1.52
Proline	0.89	0.76	0.82	0.8	0.86
Essential amino acid content	9.7	9.43	9.76	9.52	9.73
Non-essential amino acids	12	11.27	12.14	11.68	12.07
Total Amino Acid Content	21.7	20.7	21.9	21.2	21.8
Children's amino acids	2.3	2.18	2.45	2.36	2.22
Essential/Non-Essential Amino Acids	0.8083	0.8367	0.8039	0.8150	0.8061
Essential/Total Amino Acids	0.4470	0.4555	0.4456	0.4490	0.4463

Table 3. Effect of probiotics on amino acid content in broiler chicken breasts (130 days of age, 30-days of treatment).

Indexes	Groups				
	P	B	M	PB	C
15d					
Thymus Index(day15)	11.560±8.156	14.174±17.141	14.915±10.976	15.817±11.137	12.610±1.697
Spleen index(day15)	20.580±8.392	24.838±7.435	23.663±17.419	15.838±6.979	32.724±11.264
30d					
Thymus Index(day30)	27.503±21.470	8.362±3.358	19.677±20.003	22.616±20.073	10.851±16.038
Spleen index(day30)	22.145±7.127	18.445±6.461	15.856±3.742	29.427±17.904	17.347±10.956

*Different lowercase letters on the same row of data indicate significant differences ($P<0.05$), and the same or no letters indicate non-significant differences ($P>0.05$).

Table 4. Effects of probiotics on intestinal enzyme activity among testing broilers

Enzymes	Groups					
	P	B	M	PB	C	
15d						
Lipase (cecum) (ng/mL)	21.789 1.580 ab)	20.326 0.160(ab)	23.050 3.098(a)	19.046 2.70(b)	19.872 0.796(ab)	
Lipase (jejunum) (ng/mL)	21.325 3.67553(b)	23.296 0.252(ab)	24.130 0.349(ab)	26.795 3.279(a)	20.477 1.289(b)	
Chymotrypsin (cecum) (ng/mL)	16.582 0.436(ab)	16.994 1.766(ab)	18.157 1.580(a)	16.025 0.620(b)	16.095 0.567 ab)	
Chymotrypsin (jejunum) (ng/mL)	18.376 2.839	17.228 0.745	16.812 1.005	16.300 0.200	17.570 1.165	
Amylase (cecum) (ng/mL)	2.756 0.449(b)	2.447 0.140(b)	4.930 1.084(a)	3.146 0.149(b)	2.859 0.923(b)	
Amylase (jejunum) (ng/mL)	4.581 1.180(ab)	3.277 0.586(ab)	4.789 1.038(a)	3.774 0.701(ab)	3.227 0.557(b)	
30d						
Lipase (cecum) (ng/mL)	14.237 1.235(a)	11.7295 0.341 bc	10.784 0.528(b)	11.913 2.044(bc)	13.496 1.096(ac)	
Lipase (jejunum) (ng/mL)	14.339 0.792	14.387 0.6125	14.087 0.756	13.252 0.191	12.562 3.023	
Chymotrypsin (cecum) (ng/mL)	16.560 1.608(b)	15.431 0.570(b)	20.132 2.361(a)	15.351 2.374(b)	15.378 0.611(b)	
Chymotrypsin (jejunum) (ng/mL)	17.932 3.927	16.072 3.547	16.959 0.281	15.859 2.485	17.724 2.978	
Amylase (cecum) (ng/mL)	25.3462 1.670 a)	24.511 1.494(ab)	23.979 0.377(ab)	22.143 1.374(b)	25.849 2.724(a)	
Amylase (jejunum) (ng/mL)	25.426 3.321	24.521 0.992	21.426 0.460	26.235 4.729	23.749 7.0255	

*Different lowercase letters on the same row of data indicate significant differences ($P<0.05$), and the same or no letters indicate non-significant differences ($P>0.05$).

Table 5. Effects of probiotics on plasma antioxidants of broilers

Antioxidants	Groups				
	P	B	PB	M	C
15d					
MDA nmol/mL	0.979±0.481(b)	1.491±0.975(b)	1.875±1.557(b)	1.803±0.419(b)	4.379±1.697 a
SOD U/mL	25.281±1.543	24.994±0.672	22.082±4.299	25.555±0.175	25.588±0.071
GSH-Px U	352.173±498.091	252.173±232.811	310.869±134.343	230.434±112.456	110.869±53.382
T AOC U/mL	0.767±0.138(a)	0.673±0.144(ab)	0.610±0.069(ab)	0.587±0.038(ab)	0.564±0.049(b)
30d					
MDA nmol/mL	18.416±13.978(ab)	8.761±2.605(b)	7.469±5.829(b)	13.975±5.449(b)	30.154±7.520(a)
SOD U/mL	29.894±9.728(a)	22.0177±4.049(ab)	15.380±3.797(b)	20.555±2.862(ab)	22.557±0.649(ab)
GSH-Px U	1310±99.624(b)	1220±100.374(b)	1690±321.052(a)	1870±52.201(a)	1275±264.244(b)
T AOC U/mL	0.673±0.116	0.570±0.124	0.612±0.060	0.698±0.043	0.518±0.081

*Different lowercase letters on the same row of data indicate significant differences ($P<0.05$), and the same or no letters indicate non-significant differences ($P>0.05$).

*MDA Malondialdehyde SOD Superoxide dismutase GSH-Px Glutathione peroxidase T AOC Total antioxidant capacity.

Table 6. Probiotics affect changes on plasma biochemical indexes among broilers

Probiotics	Groupings				
	P	B	M	PB	C
15d					
ALB(g/L)	8.277±2.083	8.106±4.202	7.353±1.125	7.524±0.313	6.806±1.766
TP(ug/mL)	20096.057±6016.451	28829.390±8240.422	25167.025±3824.901	32773.476±14993.70	23805.376±3047.272
TG(mmol/L)	2.879±1.122(a)	1.030±0.2687(b)	0.692±0.207(b)	1.537±1.248(ab)	0.755±0.426(b)
TC(mmol/L)	4.063±1.451	3.525±1.442	2.965±0.085	4.3027±2.25884	3.698±0.274
ALT(ng/mL)	14.010±0.731	13.284±2.949	16.607±0.909	16.299±0.686	15.560± 2.493
AST(ng/mL)	21.434±7.590	16.525±1.988	18.046±0.334	25.181±4.693	76.223±95.484
30d					
ALB(g/L)	9.411±0.122	9.737±1.05376	9.981±1.729	9.940±1.039	9.207±0.577
TP(ug/mL)	39184.47±9270.572	37936.860±7231.532	43250.79±17329.705	40709.34±13747.127	36735.44±15720.88
TG(mmol/L)	0.469±0.117	2.873±4.004	1.131±0.124	0.394±0.198	0.135±0.292
TC(mmol/L)	2.449±0.362(a)	3.311±1.498(a)	2.381±0.723(a)	3.255±0.538(a)	0.208±0.035(b)
ALT(ng/mL)	13.349±2.65	12.675±1.227	11.214±0.710	11.689±1.072	10.275±2.355
AST(ng/mL)	20.848±2.436(b)	23.411±3.657(ab)	20.758±1.434(b)	22.538±0.0694(ab)	25.345±2.648(a)

*Different lowercase letters on the same row of data indicate significant differences ($P<0.05$), and the same or no letters indicate non-significant differences ($P>0.05$).

*ALB Albumin TP Total protein TG Triglycerides TC Total cholesterol ALT Phenylalanine transferase AST Aspartate transferase.

Table 7. Probiotics affects plasma immunoglobulin levels among testing broilers

Immunoglobulins	Groups				
	P	B	M	PB	C
15d					
IgM(ng/mL)	17.205± 1.155	14.255 ±2.207	15.732 ±2.219	15.965 ±1.567	16.373±1.436
IgA(ng/mL)	30.517±5.832(a)	24.521 ±2.749(a)	16.397± 4.548(b)	19.803±3.780(b)	19.190±1.248(b)
IgG(ng/mL)	7.229±2.939(ab)	3.743± 1.356(b)	8.167±0.471(a)	4.679±3.140(ab)	3.433± 1.689(b)
CD4(ng/mL)	6.534± 2.223(ab)	8.814±2.317(a)	5.329±0.951(b)	9.187±0.873(a)	6.901±1.216(ab)
CD8(ng/mL)	14.330±0.602(a)	12.825 ±1.462(ab)	12.402±1.478(ab)	11.328±1.344(b)	11.808±0.785(b)
30d					
IgM(ng/mL)	20.748±3.853	16.347±1.098	15.293±2.077	15.225±0.165	14.997±1.609
IgA(ng/mL)	46.395±1.182	45.281±2.301	41.818±2.650	39.705±2.099	41.236±3.487
IgG(ng/mL)	4.135±0.574	3.339±0.396	5.829±1.668	2.564±0.666	2.769±0.309
CD4(ng/mL)	2.500±0.409	1.195±0.574	2.267±0.501	1.622±0.346	0.425±1.076
CD8(ng/mL)	14.352±1.867(a)	11.380±3.486(a)	7.435±0.545(b)	6.141±0.332(b)	6.985±2.379(b)

*Different lowercase letters on the same row of data indicate significant differences ($P<0.05$), and the same or no letters indicate non-significant differences ($P>0.05$).

Table 8. Effect of probiotics on the morphology of jejunum in broilers

Subjects	Groupings				
	P	B	M	PB	C
15d					
the small intestinal villi length μm	10.82±1.077 b	12.53±0.813 a	11.625±1.25 ab	11.2±1.469 ab	11.05±0.737 b
intestinal gland depth μm	2.98±0.7981 b	1.82±0.286 c	2±0.216 d	2.175±0.2062 bcd	4.65±1.6823 a
VCR	3.88±1.286 c	7.088±1.511 a	5.847±0.725 a	5.158±0.633 b	2.75±1.394 cd
30d					
the small intestinal villi length μm	12.2±1.440 a	11.29±0.510 ab	10.5±0.707 b	10.00±0.816 b	10.83±0.983 b
intestinal gland depth μm	2.86±0.320 a	2.32±0.204 ab	1.3±0.308 c	1.92±0.809 bc	2.383±0.376 ab
VCR	4.353±1.039 b	4.913±0.677 b	8.354±1.593 a	6.159±3.093 b	4.638±0.813 b

*Different lowercase letters on the same row of data indicate significant differences ($P<0.05$), and the same or no letters indicate non-significant differences ($P>0.05$).

*VCR the ratio of the small intestinal villi length / intestinal gland depth

Figures

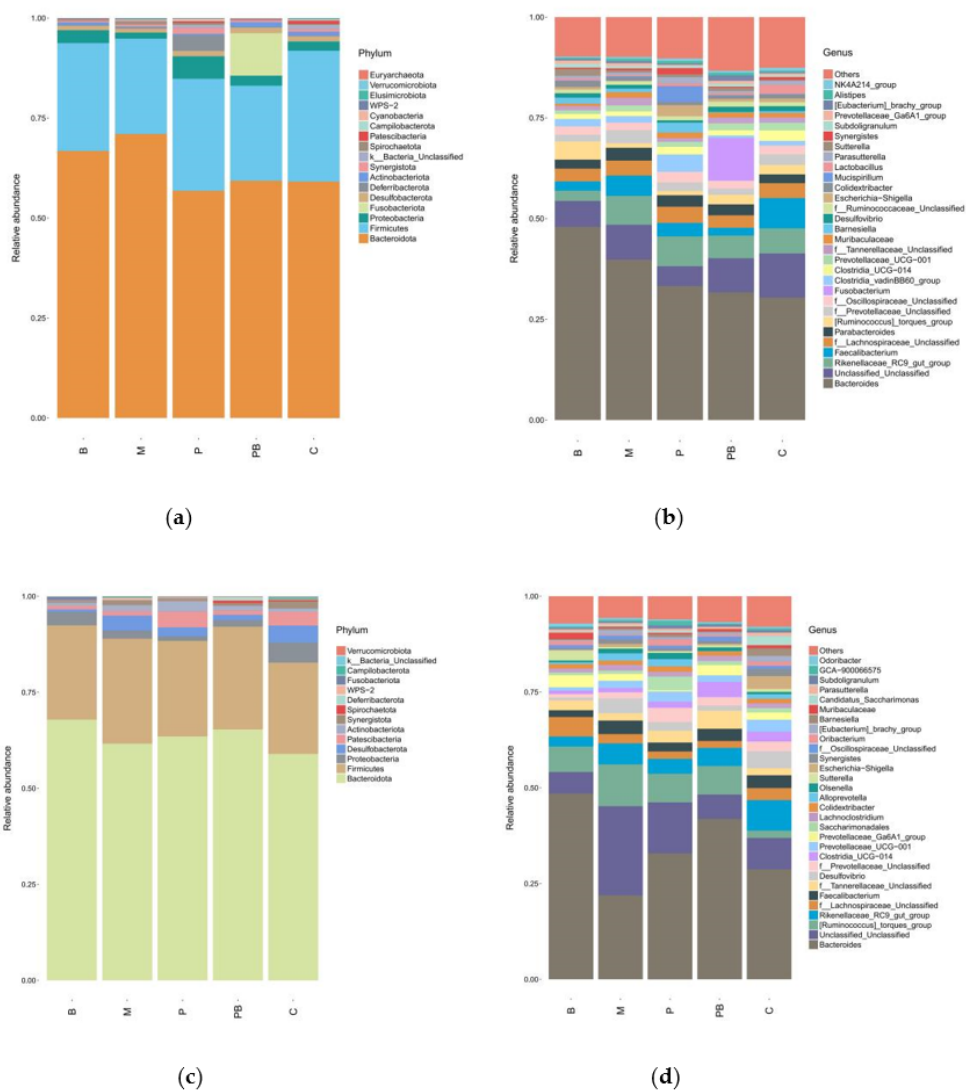


Figure 1

Bar-plot analysis of relative abundance of microbiota at the phylum and genus levels: **(a)** the phylum levels, **(b)** the genus levels (Samples collected from 15th day of the treatment); **(c)** the phylum levels, **(d)** the genus levels (Samples collected from day 30 of the treatment).