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
# Effects of nonantibiotic growth promoter combinations on growth performance, nutrient utilization, digestive enzymes, intestinal morphology, and cecal microflora of broilers

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## Abstract

Given the ban on antibiotic growth promoters, the effects of nonantibiotic alternative growth promoter combinations (NAGPCs) on the growth performance, nutrient utilization, digestive enzyme activity, intestinal morphology, and cecal microflora of broilers were evaluated. All birds were fed pellets of two basal diets—starter (0–21 d) and grower (22–42 d)—with either enramycin (ENR) or NAGPC supplemented. 1) control + ENR; 2) control diet (CON, basal diet); 3) control + mannose oligosaccharide (MOS) + mannanase (MAN) + sodium butyrate (SB) (MMS); 4) control + MOS + MAN + *Bacillus subtilis* (BS) (MMB); 5) control + MOS + fruit oligosaccharide (FOS) + SB (MFS); 6) control + FOS + BS (MFB); 7) control + MOS + FOS + MAN (MFM); 8) control + MOS + BS + phytase (PT) (MBP). ENR, MOS, FOS, SB, MAN, PT, and BS were added at 100, 2,000, 9,000, 1,500, 300, 37, and 500 mg/kg, respectively. The experiment used a completely random block design with six replicates per group: 2400 Ross 308 broilers in the starter phase and 768 in the grower phase. All NAGPCs significantly improved body weight gain ($P < 0.01$), utilization of dry matter, organic matter, and crude protein ($P < 0.05$), villus height and villus height/crypt depth in the jejunum and ileum ($P < 0.01$), and decreased the feed conversion ratio ($P < 0.01$) at d 21 and 42. MMS, MMB, MFB, and MFM duodenum trypsin, lipase, and amylase activities increased significantly ($P < 0.05$) at d 21 and 42. On d 21 and 42, MMS, MMB, and MBP increased the abundance of *Firmicutes* and *Bacteroides* whereas MMB, MFB, and MBP decreased the abundance of *Proteobacteria*, compared to ENR and CON. Overall, the NAGPCs were found to

have some beneficial effects and may be used as effective antibiotic replacements in broilers.

## Introduction

Antibiotics growth promoters (AGPs) are used in broiler diets to manage illnesses, maintain health, stimulate growth, and enhance feed utilization. However, the threats that medication resistance and antibiotic residues pose to broiler and human health have raised significant alarm regarding AGP use [1]. After the European Union and United States prohibited the use of AGPs, China followed suit in 2020. The prohibition on AGP use prompted the industry to develop suitable antibiotic replacements [2]. Growth rate reduction induced by the withdrawal of AGPs can reduce production efficiency as well as impact food safety and broiler health [3]. Therefore, in the absence of AGPs, alternate methods need to be developed to ensure feed efficiency and broiler health [4].

As a result of the ban on antibiotic growth promoters in broiler production, broilers have become susceptible to enteric diseases and reduced growth performance and nutrient digestibility. This is due to the invasion of intestinal pathogens, intestinal injury, decrease in the activity of intestinal digestive enzymes, and the effect of anti-nutritional factors in feed. This poses a serious challenge for boiler production. Therefore, many research studies have been conducted to find potential replacements for dietary antibiotics to address these issues, ensure the health of broilers, and increase the efficiency of their productivity [5–7].

Currently, nonantibiotic alternative growth promoters (NAGPs) such as enzymes, probiotics, prebiotics, and acidifiers are being used in broiler feed to replace antibiotics [8]. As the energy source of intestinal bacteria, butyric acid in sodium butyrate (SB) can improve the intestinal flora and its structure and nutrient digestibility [9]. The digestive capacity of the intestinal tract can be improved using the digestive enzymes released by *Bacillus subtilis* (BS) [10]. By preventing colonization of intestinal pathogenic bacteria, prebiotics dominated by mannose oligosaccharide (MOS) can also improve the intestinal environment [11]. Mannanase (MAN) can hydrolyze the non-starch polysaccharide present in soya bean meal and supplement endogenous enzymes to increase nutrient digestibility [12]. Owing to its unique spatial structure, phytase (PT) can breakdown phytic acid into inositol and inorganic phosphorus and promote the release of other nutrients along with phytic acid [13]. The different mechanisms and modes of action of these enzymes, probiotics, prebiotics, and organic acids suggest the potential for complementary synergistic effects upon supplementing diets with a mixture of these additives.

In a previous study comparing the effects of formic acid, propionic acid, and their combination on the growth performance of broilers, the feed conversion ratio for only formic acid + propionic acid dropped significantly compared with that for the other combinations [14]. Another study on poultry diets showed that a combination of xylanase, amylase, and protease could elicit greater improvements in nutrient utilization than xylanase, amylase, or protease given alone [15]. These results indicate that NAGPCs have better effect than NAGPs alone. However, current research on NAGPSs is mainly focused on the effect of combining the same kinds of NAGPs in broilers. The beneficial effect of synergy may be limited by the combination of the same kind of NAGPs.

Therefore, the objective of this study was to evaluate the effects of six NAGPCs with different types of NAGPs on growth performance, nutrient utilization, digestive enzyme activity, intestinal morphology, and cecal microflora of broilers so as to find a better alternative method to ensure feed efficiency and the broiler health.

## Materials and methods

### Ethics approval

Animal care and experimental protocols (License no. QAU20210607) were approved by the Animal Care and Use Committee of Qingdao Agricultural University, China. The animals were cared for according to the Animal Care Guidelines of China. All efforts were made to minimize animal suffering. All broilers could get appropriate housing conditions and adequate food and water. The cervical dislocation method was used to euthanize the broilers. All personnel involved in animal care and euthanasia were trained and certified in accordance with the guidelines of the Animal Care Committee of Qingdao Agricultural University.

### Birds, housing and management

In the starter phases, 2,400 1-d-old healthy Ross 308 broilers of both sexes with similar body weight (BW) were randomly assigned to eight groups; each group included six repeats with 50 chickens each. In the grower phases, 768 21-d-old healthy Ross 308 broilers with similar BW were randomly assigned to eight groups; each group included six repeats with 16 chickens each. The feeding experiment was conducted for 21 d after individual weighing. No significant differences in the initial BW were observed among these groups. Seven-day-old chickens were immunized with live Newcastle disease (150132007; Yibang Biological Engineering Co., Ltd., Qingdao, China) and infectious bronchitis vaccines (150132016; Yibang Biological Engineering Co., Ltd., Qingdao, China) by nose and eye dripping. Fourteen-day-old chickens were immunized with a live infectious bursal disease vaccine (150132026; Yibang Biological Engineering Co., Ltd., Qingdao, China) in drinking water. At 21 d of age, the chickens were immunized with a live Newcastle disease vaccine (150132007; Yibang Biological Engineering Co., Ltd., Qingdao, China) by nose and eye dripping. During the early stages of the experiment (0–21 days), 50 broilers in each replicate were reared in a single cage (130 cm × 55 cm × 50 cm). During the latter stages of the experiment (22–42 days), each replicate was established by housing 16 birds per cage. The broilers had free access to pellet feed and water throughout the study. In a controlled environment with a relative humidity of 45–55% and temperature of 25–34°C, the broilers were maintained under an 18 h/6 h light/dark cycle. In the first week of the experiment, the ambient temperature was maintained at 34°C; it was then gradually decreased to 26°C after 21 d and maintained thereafter.

### Experimental design and diets

The NAGPs used in this study are the most commonly used in broiler farms; their addition levels for the *in vitro* digestion test were determined using meta-analysis. The selection of the amounts of different NAGPs and NAGPCs to be added was based on the in vitro digestion test, which followed the research method established by Graham et al. [16] and Pedersen et al. [17]. All broilers were fed pellets of two basal diets (Table 1)—starter (0–21 d) or grower (22–42 d)—that differed within each phase by the addition of either enramycin (ENR) or NAGPCs, as follows: 1) control + 100 mg/kg ENR; 2) a control diet (CON, basal diet); 3) control + 2,000 mg/kg MOS + 300 mg/kg MAN + 1,500 mg/kg SB (MMS); 4) control + 2,000 mg/kg MOS + 300 mg/kg MAN + 500 mg/kg BS (MMB); 5) control + 2,000 mg/kg MOS + 9,000 mg/kg FOS + 1,500 mg/kg SB (MFS); 6) control + 2,000 mg/kg MOS + 9,000 mg/kg FOS + 500 mg/kg BS (MFB); 7) control + 2,000 mg/kg MOS + 9,000 mg/kg FOS + 300 mg/kg MAN (MFM); and 8) control + 2,000 mg/kg MOS + 500 mg/kg BS + 37 mg/kg PT (MBP). SB ($\geq$ 50%) was obtained from Qilu Animal Health Co., Ltd. MOS ($\geq$ 12%) was obtained from Orteki Biological products Co., Ltd. Fruit oligosaccharide (FOS, $\geq$ 95%) was obtained from Baolingbao

**Table 1. Ingredient (%) and nutritive value of a basal diet.**

| Item | Starter feed | Grower feed |
|---|---|---|
| Ingredient (%) | | |
| Corn | 45.00 | 44.30 |
| Wheat | 15.00 | 14.70 |
| Expanded soybean meal | 23.50 | 22.00 |
| Cottonseed meal | 5.00 | 5.00 |
| Corn gluten meal | 2.50 | 1.00 |
| Hydrolyzed feather meal | 1.50 | 1.00 |
| Calcium bicarbonate phosphate | 0.80 | 0.60 |
| Stone powder | 1.70 | 1.70 |
| Bentonite | 0.50 | 0.50 |
| Soybean oil | 2.20 | 7.00 |
| Sodium chloride | 0.25 | 0.25 |
| Lysine | 0.65 | 0.62 |
| Methionine | 0.25 | 0.23 |
| Threonine | 0.15 | 0.10 |
| Premix[1] | 1.00 | 1.00 |
| Total | 100.00 | 100.00 |
| Nutrition level[2] | | |
| ME (kcal/kg) | 2900 | 3200 |
| CP (%) | 22.00 | 20.00 |
| Calcium (%) | 0.98 | 0.88 |
| Total phosphorus (%) | 0.76 | 0.65 |
| Lysine (%) | 1.41 | 1.37 |
| Methionine (%) | 0.56 | 0.51 |
| Threonine (%) | 0.91 | 0.80 |

[1]Vitamin-mineral premix (each kg contained); vitamin A, 9050 IU; vitamin D3, 1950 IU; vitamin E, 26 IU; vitamin K3, 5.0 mg; vitamin B1, 2.6 mg; vitamin B2, 8.0 mg; pantothenic acid, 15.0 mg; vitamin B6,3.0 mg; vitamin B12, 0.02 mg; nicotinic acid, 35 mg; choline chloride, 9050 mg; biotin, 0.20 mg; folic acid, 1.2 mg; Mn,60 mg. Fe,80 mg; Zn,60 mg; Cu,8.5 mg; I,0.27 mg; Se,0.20 mg

[2]Nutrient levels were calculated by analysis.

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Biological Co., Ltd. MAN ($\geq$ 50,000 U/g) was obtained from Shandong Shengdao Biotechnology Co., Ltd. PT ($\geq 3 \times 10^5$ U/g) was obtained from Jinan Baisijie Biological Engineering Co., Ltd. BS ($\geq 2 \times 10^{11}$ cfu/g) was obtained from Beijing Keweibo Biotechnology Co., Ltd. The products were added manually, and no specific premix or carrier was used. The experimental materials were all products available on the market. The MMS diet was composed of 240 mg/kg of MOS, 15,000 U/kg of MAN, and 750 mg/kg of SB. The MMB diet was composed of 240 mg/kg of MOS, 15,000 U/kg of MAN, and $1 \times 10^{11}$ cfu/kg of BS. The MFS diet was composed of 240 mg/kg of MOS, 8,550 mg/kg of FOS, and 750 mg/kg of SB. The MFB diet was composed of 240 mg/kg of MOS, 8,550 mg/kg of FOS, and $1 \times 10^{11}$ cfu/kg of BS. The MFM diet was composed of 240 mg/kg of MOS, 8,550 mg/kg of FOS, and 15,000 U/kg of MAN. The MBP diet was composed of 240 mg/kg of MOS, $1 \times 10^{11}$ cfu/kg of BS, and 11,100 U/kg of PT. The following pellet conditioning parameters were applied: maximum steam injection at 1.3 kgf, 2.7% water addition, and 60°C temperature. All diets were formulated based on the nutrient requirement of poultry published by the National Research Council [18] and met the nutrient requirements of broiler chickens. The ingredients and nutrient levels of the diets are listed in

Table 1. Broilers were weighed individually at 1, 21, and 42 days of age. The feed intake was recorded in replicate at the same time intervals and the feed conversion ratio (FCR) was calculated on a replicate basis. At d 21 and 42, one broiler from each replicate (six per treatment) was euthanized by cervical dislocation.

## Sample collection

**Nutrient utilization.** Feed samples of 500 g were collected by quartering at the beginning of each stage. The excreta sample of each repeat was collected from days 19 to 21 and days 40 to 42. On the last day of collection, the pooled excreta were mixed well using clean glass sticks. Representative samples of 200 g were taken from each replicate. The representative samples were then lyophilized, ground, and passed through a 0.5 mm screen. Excreta and diet samples were analyzed for dry matter (DM) (AOAC Official method 930.15, 2006) [19], ash (AOAC Official method 942.05, 2006) [19], crude protein (CP) (AOAC Official method 990.02, 1990) [20], ether extract (AOAC Official method 968.06, 2000) [21], calcium (Ca) and phosphorus (P) (AOAC Official method 2011.14, 1990) [20], and acid insoluble ash (AIA) (AOAC Official method 975.12, 1990) [20]. The ether extract was crude fat (CF). Nutrient utilization was determined using AIA as an indicator. Feed and excreta were corrected based on DM. Nutrient utilization was calculated from the following equation:

$$\text{Nutrient utilization (\%)} = 1 - ([\%\text{nutrient}_{\text{excreta}}/\%\text{nutrient}_{\text{diet}}] \times [\%\text{AIA}_{\text{diet}}/\%\text{AIA}_{\text{excreta}}]) \times 100\%$$

where nutrient indicates the nutrient content in feed or excreta, and AIA indicates acid insoluble ash content in feed or excreta.

**Measurement of digestive enzyme activity.** Following euthanasia, one gram of duodenal content was removed using an aseptic scraper and placed in a 2 mL aseptic tube that was frozen at -20˚C. Amylase, lipase, and trypsin activity in the duodenum was determined using corresponding diagnostic kits (amylase C016-1, lipase A054-1, trypsin A080-2; Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

**Villus histomorphometry.** Following euthanasia, the mid-jejunum and mid-ileum segments were removed (about 2 cm). After emptying their contents with distilled water using a Nalgene LDPE wash bottle, each tissue was individually fixed in a formalin solution and stored at room temperature. The jejunum was then used to measure the intestinal villi and crypts. The small intestine samples (jejunum and ileum) were fixed in 4% buffered formaldehyde and then dehydrated using a graded series of xylene and ethanol before embedding in paraffin for histological analysis. The small intestine sections (8 microns in length) were then deparaffinized using xylene and rehydrated using graded ethanol dilutions. Hematoxylin and eosin were used to stain the slides. Ten slides were prepared for each sample (from the central region of the sample), and images were captured using an optical binocular microscope (OLYMPUS CKX53, Jingkai instrument and equipment Co., Ltd., Suzhou, China). The villus height and crypt depth were measured by Image—Pro Plus 6. 0 five times from distinct villi and crypts on each slide. Averages were computed for these values.

**16S rDNA sequencing and data analysis.** After euthanasia, one gram of cecal content was removed using an aseptic scraper and placed in a 2 mL aseptic tube that was frozen at -20˚C. Total genomic DNA from cecal digesta was extracted using PowerSoil® DNA Isolation Kit (Sigma-Aldrich, Carlsbad, United States of America). DNA concentration and purity were assessed using a Synergy HTX (Gene Company Limited, Shanghai, China). DNA amplicons from individual samples were amplified using polymerase chain reaction with specific primers for the V3-V4 regions of the 16S rRNA gene. Amplicons generated from each sample were

subjected to agarose (1%) gel electrophoresis, excised, purified using a Monarch DNA kit (Jizhi biochemical Technology Co., Ltd., Shanghai, China), and quantified using the Synergy HTX system. The constructed library was sequenced on the Illumina Novaseq6000 PE250 platform (Jingneng Biotechnology Co., Ltd., Shanghai, China). The original data were spliced (FLASH, version 1.2.11); the spliced sequences were filtered by quality (Trimmomatic, version 0.33), and chimerism (UCHIME, version 8.1) was removed to obtain high-quality tag sequences. The sequences were clustered at the level of 97% similarity using USEARCH (version 10.0). By default, 0.005% of all sequenced sequences was taken as the threshold for filtering operational taxonomic units (OTUs). Alpha diversity analysis was performed using a classifier Bayesian algorithm (https://qiime2.org/) and the QIIME 2 software. Principal coordinate analysis (PCoA) and beta diversity analysis implemented in the QIIME 2 software were performed based on UniFrac distance matrices. Linear discriminant analysis Effect Size (LEFSe) used Kruskal-Wallis rank sum to detect species with significant abundance differences between groups. The Wilcoxon rank-sum test was then used to determine the consistency of the differences between different subgroups. Finally, linear regression analysis (LDA) was used to estimate the magnitude of the influence of the abundance of each component (species) on the differential effect. Color correlograms were generated using the R corrplot package (Version 0.84).

## Statistical analyses

All data were analyzed using one-way analysis of variance (ANOVA) and the *Tukey's Honest Standard Difference test* using the SPSS statistical software (version 20.0; SPSS Inc., Chicago, IL, USA). A P value < 0.05 was considered to indicate statistical significance. The results are expressed as the mean and pooled standard error of mean (SEM). The experimental units were replicates and the statistical model was as follows:

$$Y_{ij} = \mu + A_i + e_{ij}$$

where $Y_{ij}$ represents an observation, $\mu$ is the overall mean, $A_i$ represents the effect of NAGPCs, and $e_{ij}$ represents random error.

## Results

### Growth performance

There were no significant differences in initial body weight (Table 2). On d 21 and 42, body weight gain (BWG) increased significantly (P < 0.01) in the MMS, MMB, MFS, MFB, MFM, and MBP groups compared with that in the CON and ENR groups. On d 21 and 42, FCR decreased significantly (P < 0.01) in the MMS, MMB, MFS, MFB, MFM, and MBP groups compared with that in the CON and ENR groups.

### Nutrient utilization

On d 21, DM, OM, CP, and CF utilization increased significantly (P < 0.01) in the MMS, MMB, MFS, MFB, MFM, and MBP groups compared with that in the CON and ENR groups, whereas the treatments had no effect on Ca (P = 0.11) and P (P = 0.1) utilization (Table 3). On d 42, a significant increase was observed in DM, OM, CP, and CF (P < 0.01) utilization in the MMS, MMB, MFS, MFB, MFM, and MBP groups compared with that in the CON and ENR groups. On d 42, a significant increase in Ca (P < 0.01) and P (P < 0.01) utilization was found in the ENR, MMS, MMB, MFS, MFB, MFM, and MBP groups compared with that in the CON group.

**Table 2. Effects of dietary nonantibiotic alternative growth promoter combinations on body weight gain and feed conversion ratio of broilers at 21 and 42 days.**

| Items[1] | ENR | CON | MMS | MMB | MFS | MFB | MFM | MBP | SEM | p |
|---|---|---|---|---|---|---|---|---|---|---|
| BW (g) | | | | | | | | | | |
| 1 day of age | 40.4 | 40.17 | 40.62 | 39.82 | 40.44 | 40.54 | 40.25 | 40.58 | 0.07 | >0.05 |
| 22 day of age | 985.10 | 993.65 | 966.25 | 950.52 | 1053.18 | 972.35 | 1053.80 | 1059.27 | 18.86 | >0.05 |
| BWG (g) | | | | | | | | | | |
| 1–21 day of age | 791.26[d] | 746.03[e] | 941.61[a] | 896.01[c] | 919.88[b] | 938.05[a] | 903.03[c] | 919.45[b] | 9.92 | <0.01 |
| 22–42 day of age | 2057.46[e] | 1919.84[f] | 2094.49[d] | 2187.62[a] | 2139.18[b] | 2111.47[c] | 2148.20[b] | 2102.17[cd] | 11.07 | <0.01 |
| FCR | | | | | | | | | | |
| 1–21 day of age | 1.243[b] | 1.287[a] | 1.196[de] | 1.190[e] | 1.217[c] | 1.207[cd] | 1.206[cd] | 1.211[cd] | 0.00 | <0.01 |
| 22–42 day of age | 1.723[a] | 1.658[b] | 1.485[e] | 1.443[g] | 1.541[c] | 1.46[f] | 1.514[d] | 1.532[c] | 0.01 | <0.01 |

[a,b]Values within the same row with different superscripts were significantly different (P < 0.05).

[1]CON, control diet; ENR; CON + 100 mg/kg ENR, MMS; CON + 2000 mg/kg MOS + 300 mg/kg MAN + 1500 mg/kg SB, MMB; CON + 2000 mg/kg MOS + 300 mg/kg MAN + 500 mg/kg BS, MFS; CON + 2000 mg/kg MOS + 9000 mg/kg FOS + 1500 mg/kg SB, MFB; CON + 2000 mg/kg MOS + 9000 mg/kg FOS + 500 mg/kg BS, MFM; CON + 2000 mg/kg MOS + 9000 mg/kg FOS + 300 mg/kg MAN, MBP; CON + 2000 mg/kg MOS + 500 mg/kg BS + 37 mg/kg PT. SB; Sodium butyrate, FOS; Fructose oligosaccharide, PT; Phytase, BS; Bacillus subtilis, MOS; Mannose oligosaccharide, MAN; Mannanase, BW; body weight, BWG; body weight gain, FCR; feed conversion ratio.

## Duodenal digestive enzyme activity

On d 21, a significant increase in the activity of duodenal trypsin (P < 0.01) was observed in the MMS, MMB, MFB, and MFM groups compared with that in the CON and ENR groups (Table 4). There was also an increase in the activity of duodenal lipase (P < 0.01) in the MMS,

**Table 3. Effects of dietary nonantibiotic alternative growth promoter combinations on the utilization of dry matter (DM), organic matter (OM), crude protein (CP), crude fat (CF), calcium (Ca), and phosphorus (P) in broiler chicks at 21 and 42 days.**

| Items[1] (%) | ENR | CON | MMS | MMB | MFS | MFB | MFM | MBP | SEM | p |
|---|---|---|---|---|---|---|---|---|---|---|
| 21d | | | | | | | | | | |
| DM | 75.66[f] | 74.59[g] | 78.27[a] | 78.28[a] | 77.19[e] | 77.94[c] | 78.11[b] | 77.49[d] | 0.19 | <0.01 |
| OM | 68.44[d] | 67.25[e] | 73.43[a] | 73.48[a] | 71.4[c] | 71.7[b] | 71.33[c] | 71.55[bc] | 0.30 | <0.01 |
| CP | 65.68[c] | 64.97[c] | 71.29[ab] | 71.33[a] | 70.39[b] | 71.2[ab] | 71.26[ab] | 70.55[ab] | 0.37 | <0.01 |
| CF | 67.01[d] | 64.91[e] | 74.47[ab] | 74.72[a] | 73.45[c] | 74.59[ab] | 74.32[ab] | 74.02[bc] | 0.53 | <0.01 |
| Ca | 65.14 | 65.13 | 65.4 | 65.74 | 65.26 | 65.66 | 65.09 | 67.53 | 0.22 | 0.11 |
| P | 63.84 | 62.9 | 65.34 | 65.5 | 65.17 | 65.75 | 65.55 | 65.67 | 0.28 | 0.10 |
| 42d | | | | | | | | | | |
| DM | 76.53[g] | 74.86[h] | 80.04[c] | 80.56[a] | 78.22[f] | 80.37[b] | 79.72[d] | 78.76[e] | 0.28 | <0.01 |
| OM | 72.03[f] | 67.81[g] | 75.16[bc] | 75.53[a] | 73.22[e] | 75.22[b] | 75.01[c] | 73.46[d] | 0.35 | <0.01 |
| CP | 69.7[e] | 67.72[f] | 72.59[b] | 73.29[a] | 70.25[d] | 73.28[a] | 72.02[c] | 70.52[d] | 0.27 | <0.01 |
| CF | 71.25[d] | 69.12[e] | 76.27[ab] | 76.84[a] | 72.13[c] | 76.53[a] | 75.75[b] | 72.06[c] | 0.41 | <0.01 |
| Ca | 65.62[a] | 62.49[b] | 66.44[a] | 66.67[a] | 66.29[a] | 67.21[a] | 66.14[a] | 67.39[a] | 0.25 | <0.01 |
| P | 67.87[c] | 64.59[d] | 68.37[bc] | 68.49[b] | 68.34[bc] | 68.42[b] | 68.33[bc] | 69.21[a] | 0.20 | <0.01 |

[a,b]Values within the same row with different superscripts were significantly different (P < 0.05).

[1]CON, control diet; ENR; CON + 100 mg/kg ENR, MMS; CON + 2000 mg/kg MOS + 300 mg/kg MAN + 1500 mg/kg SB, MMB; CON + 2000 mg/kg MOS + 300 mg/kg MAN + 500 mg/kg BS, MFS; CON + 2000 mg/kg MOS + 9000 mg/kg FOS + 1500 mg/kg SB, MFB; CON + 2000 mg/kg MOS + 9000 mg/kg FOS + 500 mg/kg BS, MFM; CON + 2000 mg/kg MOS + 9000 mg/kg FOS + 300 mg/kg MAN, MBP; CON + 2000 mg/kg MOS + 500 mg/kg BS + 37 mg/kg PT. SB; Sodium butyrate, FOS; Fructose oligosaccharide, PT; Phytase, BS; Bacillus subtilis, MOS; Mannose oligosaccharide, MAN; Mannanase, DM; dry matter, OM; organic matter, CP; crude protein, CF; crude fat, Ca; calcium, P; phosphorus.

**Table 4. Effects of dietary nonantibiotic alternative growth promoter combinations on duodenal digestive enzyme activity of broilers at 21 and 42 days.**

| Parameters [1] (U/mg protein) | ENR | CON | MMS | MMB | MFS | MFB | MFM | MBP | SEM | p |
|---|---|---|---|---|---|---|---|---|---|---|
| 21d | | | | | | | | | | |
| trypsin | 1863.94[cd] | 1580.58[d] | 2512.92[ab] | 2603.89[a] | 2154.42[bc] | 2350.65[ab] | 2416.65[ab] | 2266.59[abc] | 64.51 | <0.01 |
| lipase | 55.4[d] | 52.49[d] | 66.22[ab] | 66.55[a] | 62.08[bc] | 62.14[bc] | 61.04[bc] | 61.02[bc] | 0.86 | <0.01 |
| amylase | 7.44[bc] | 5.71[c] | 9.52[ab] | 9.98[a] | 8.26[ab] | 8.23[ab] | 8.58[ab] | 8.16[abc] | 0.27 | <0.01 |
| 42d | | | | | | | | | | |
| trypsin | 1876.3[c] | 1621[c] | 2730.78[ab] | 2842.87[a] | 2336.71[b] | 2828.67[a] | 2696.65[ab] | 2491.59[ab] | 81.27 | <0.01 |
| lipase | 64.88[c] | 59.77[d] | 75.65[ab] | 76.47[a] | 71.68[b] | 76.3[ab] | 74.28[ab] | 71.74[b] | 1.06 | <0.01 |
| amylase | 7.44[c] | 6.13[d] | 9.25[ab] | 9.53[a] | 8.17[bc] | 9.38[ab] | 9.21[ab] | 8.24[abc] | 0.22 | <0.01 |

[a,b]Values within the same row with different superscripts were significantly different (P < 0.05).

[1]CON, control diet; ENR; CON + 100 mg/kg ENR, MMS; CON + 2000 mg/kg MOS + 300 mg/kg MAN + 1500 mg/kg SB, MMB; CON + 2000 mg/kg MOS + 300 mg/kg MAN + 500 mg/kg BS, MFS; CON + 2000 mg/kg MOS + 9000 mg/kg FOS + 1500 mg/kg SB, MFB; CON + 2000 mg/kg MOS + 9000 mg/kg FOS + 500 mg/kg BS, MFM; CON + 2000 mg/kg MOS + 9000 mg/kg FOS + 300 mg/kg MAN, MBP; CON + 2000 mg/kg MOS + 500 mg/kg BS + 37 mg/kg PT. SB; Sodium butyrate, FOS; Fructose oligosaccharide, PT; Phytase, BS; Bacillus subtilis, MOS; Mannose oligosaccharide, MAN; Mannanase.

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MMB, MFS, MFB, MFM, and MBP groups. Compared with that of the CON and ENR groups, a significant increase in duodenal amylase activity was also observed in the MMB group. On d 42, a significant increase in the duodenal trypsin and lipase (P < 0.01) activity in the MMS, MMB, MFS, MFB, MFM, and MBP groups was observed compared with that in the CON and ENR groups; furthermore, a significant increase in duodenal amylase (P < 0.01) activity was observed in the MMS, MMB, MFB, and MFM groups.

## Ileum and jejunum histology

On d 21, the villus height (P < 0.01) in the jejunum were significantly increased in the MMS, MMB, MFS, MFB, MFM, and MBP groups compared with those in the CON and ENR groups and villus height/crypt depth (P < 0.01) in the jejunum were significantly increased in the MMS, MMB, MFB, and MFM groups (Table 5, Figs 1–4). Compared with those in the CON and ENR groups, the ileal villus height (P < 0.01) and villus height/crypt depth (P < 0.01) were significantly increased in the MMB group and crypt depth (P < 0.01) was significant decreased in the MMS, MMB, MFS, MFB, MFM, and MBP groups. On d 42, the villus height (P < 0.01) and villus height/crypt depth (P < 0.01) in the jejunum were significantly increased in the MMS, MMB, MFS, MFB, MFM, and MBP groups compared with those in the CON and ENR groups; crypt depth (P < 0.01) was also significantly decreased. Ileal villus height (P < 0.01) was significantly increased in the MMS, MMB, MFS, MFB, MFM, and MBP groups compared with that in the CON and ENR groups; furthermore, the crypt depth (P < 0.01) showed a significant decrease, whereas the villus height/crypt depth ratio (P < 0.01) showed a significant increase.

## Composition and differences of cecal microflora

**Phylum level.** Fig 5A and 5B show the relative abundance of the top 10 microorganisms at the phylum level in 21- and 42-d-old birds. The cecal microbiome of each group at 21 d was dominated by *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Tenericutes*, *Verrucomicrobia*, *Actinobacteria*, *Cyanobacteria*, *Acidobacteria*, *Fusobacteria*, and *Epsilonbacteraeota*. The cecal microbiome of each group at 42 d of age was dominated by *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Tenericutes*, *Lentisphaerae*, *Actinobacteria*, *Cyanobacteria*, *Acidobacteria*, *Fusobacteria*, and

**Table 5. Effects of dietary nonantibiotic alternative growth promoter combinations on ileal and jejunum histomorphological parameters of broilers at 21 and 42 days.**

| Items[1] | ENR | CON | MMS | MMB | MFS | MFB | MFM | MBP | SEM | p |
|---|---|---|---|---|---|---|---|---|---|---|
| 21d | | | | | | | | | | |
| jejunum | | | | | | | | | | |
| villus height (μm) | 562.21[c] | 320.43[d] | 922.07[a] | 939.13[a] | 681.10[b] | 911.74[a] | 905.55[a] | 690.28[b] | 43.72 | <0.01 |
| crypt depth (μm) | 105.18 | 118.22 | 93.30 | 92.95 | 96.05 | 93.54 | 94.18 | 96.54 | 2.66 | 0.19 |
| villus height/crypt depth | 5.39[b] | 2.77[c] | 10.00[a] | 10.18[a] | 7.09[b] | 9.77[a] | 9.70[a] | 7.15[b] | 0.54 | <0.01 |
| ileal | | | | | | | | | | |
| villus height (μm) | 486.82[c] | 435.45[c] | 845.27[ab] | 873.05[a] | 792.17[b] | 827.22[ab] | 812.34[ab] | 805.29[ab] | 33.67 | <0.01 |
| crypt depth (μm) | 119.20[a] | 126.76[a] | 89.47[b] | 85.61[b] | 91.96[b] | 89.44[b] | 90.06[b] | 91.51[b] | 3.14 | <0.01 |
| villus height/crypt depth | 4.09[c] | 3.44[c] | 9.45[ab] | 10.20[a] | 8.62[b] | 9.24[b] | 9.01[b] | 8.82[b] | 0.51 | <0.01 |
| 42d | | | | | | | | | | |
| jejunum | | | | | | | | | | |
| villus height (μm) | 802.48[b] | 698.15[b] | 1133.07[a] | 1159.25[a] | 1081.09[a] | 1188.26[a] | 1131.01[a] | 1107.43[a] | 37.32 | <0.01 |
| crypt depth (μm) | 123.15[a] | 137.60[a] | 81.18[b] | 80.67[b] | 87.15[b] | 80.34[b] | 84.06[b] | 85.72[b] | 4.48 | <0.01 |
| villus height/crypt depth | 6.55[b] | 5.10[b] | 14.03[a] | 14.36[a] | 12.40[a] | 14.79[a] | 13.54[a] | 12.91[a] | 0.75 | <0.01 |
| ileal | | | | | | | | | | |
| villus height (μm) | 648.73[d] | 571.34[e] | 1249.72[ab] | 1273.86[a] | 988.50[c] | 1271.14[a] | 1187.20[b] | 993.01[c] | 54.80 | <0.01 |
| crypt depth (μm) | 130.43[b] | 144.66[a] | 77.77[cd] | 73.67[d] | 89.01[c] | 76.08[cd] | 78.54[cd] | 83.33[c] | 5.41 | <0.01 |
| villus height/crypt depth | 4.98[d] | 3.95[d] | 16.08[ab] | 17.29[a] | 11.11[c] | 16.72[ab] | 15.15[b] | 11.92[c] | 1.02 | <0.01 |

[a,b]Values within the same row with different superscripts were significantly different (P < 0.05).

[1]CON, control diet; ENR; CON + 100 mg/kg ENR, MMS; CON + 2000 mg/kg MOS + 300 mg/kg MAN + 1500 mg/kg SB, MMB; CON + 2000 mg/kg MOS + 300 mg/kg MAN + 500 mg/kg BS, MFS; CON + 2000 mg/kg MOS + 9000 mg/kg FOS + 1500 mg/kg SB, MFB; CON + 2000 mg/kg MOS + 9000 mg/kg FOS + 500 mg/kg BS, MFM; CON + 2000 mg/kg MOS + 9000 mg/kg FOS + 300 mg/kg MAN, MBP; CON + 2000 mg/kg MOS + 500 mg/kg BS + 37 mg/kg PT. SB; Sodium butyrate, FOS; Fructose oligosaccharide, PT; Phytase, BS; Bacillus subtilis, MOS; Mannose oligosaccharide, MAN; Mannanase.
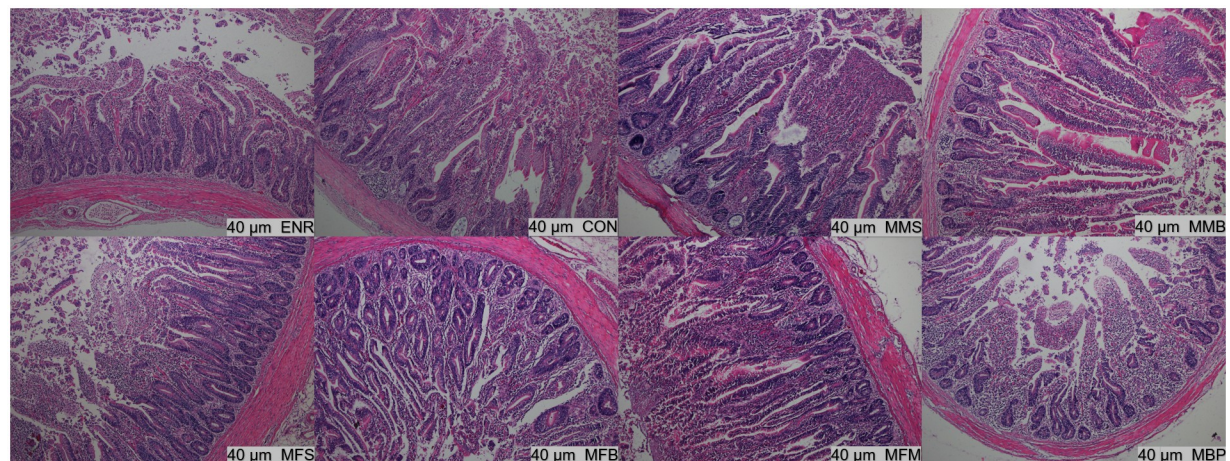
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**Fig 1. Histological structure of Hematoxylin and Eosin (H&E) staining jejunum at 21 day of age.** CON, control diet; ENR; CON + 100 mg/kg ENR, MMS; CON + 2000 mg/kg MOS + 300 mg/kg MAN + 1500 mg/kg SB, MMB; CON + 2000 mg/kg MOS + 300 mg/kg MAN + 500 mg/kg BS, MFS; CON + 2000 mg/kg MOS + 9000 mg/kg FOS + 1500 mg/kg SB, MFB; CON + 2000 mg/kg MOS + 9000 mg/kg FOS + 500 mg/kg BS, MFM; CON + 2000 mg/kg MOS + 9000 mg/kg FOS + 300 mg/kg MAN, MBP; CON + 2000 mg/kg MOS + 500 mg/kg BS + 37 mg/kg PT. SB; Sodium butyrate, FOS; Fructose oligosaccharide, PT; Phytase, BS; Bacillus subtilis, MOS; Mannose oligosaccharide, MAN; Mannanase.
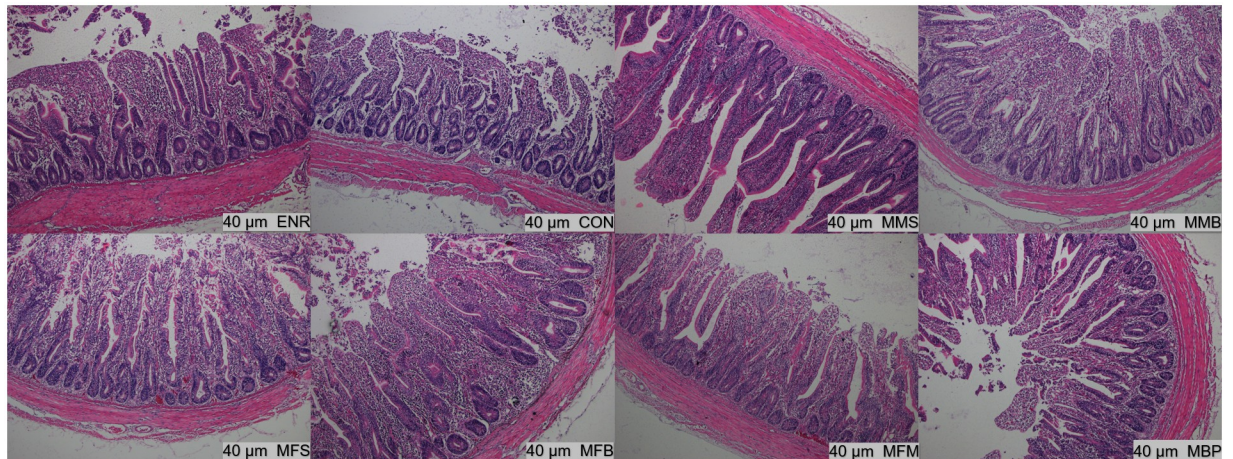
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**Fig 2. Histological structure of Hematoxylin and Eosin (H&E) staining ileum at 21 day of age.** CON, control diet; ENR; CON + 100 mg/kg ENR, MMS; CON + 2000 mg/kg MOS + 300 mg/kg MAN + 1500 mg/kg SB, MMB; CON + 2000 mg/kg MOS + 300 mg/kg MAN + 500 mg/kg BS, MFS; CON + 2000 mg/kg MOS + 9000 mg/kg FOS + 1500 mg/kg SB, MFB; CON + 2000 mg/kg MOS + 9000 mg/kg FOS + 500 mg/kg BS, MFM; CON + 2000 mg/kg MOS + 9000 mg/kg FOS + 300 mg/kg MAN, MBP; CON + 2000 mg/kg MOS + 500 mg/kg BS + 37 mg/kg PT. SB; Sodium butyrate, FOS; Fructose oligosaccharide, PT; Phytase, BS; Bacillus subtilis, MOS; Mannose oligosaccharide, MAN; Mannanase.

*Epsilonbacteraeota*. Among these, *Bacteroidetes* and *Firmicutes* were the most dominant bacterial groups, which together accounted for more than 80% of the total microbial community detected. Fig 5C and 5D show compared with the ENR and CON groups, the MMS, MMB, and MBP groups showed increase in the abundance of *Firmicutes* at 21 d and in the abundance of *Bacteroides* at 42 d, whereas the MMB, MFB, and MBP groups showed decreased abundance of *Proteobacteria* at 21 and 42 d.

**Genus level.** Fig 6A and 6B show the relative abundance of the top 10 microorganisms at the genus level in 21- and 42-d-old birds. Fig 6C and 6D show compared with that in the CON
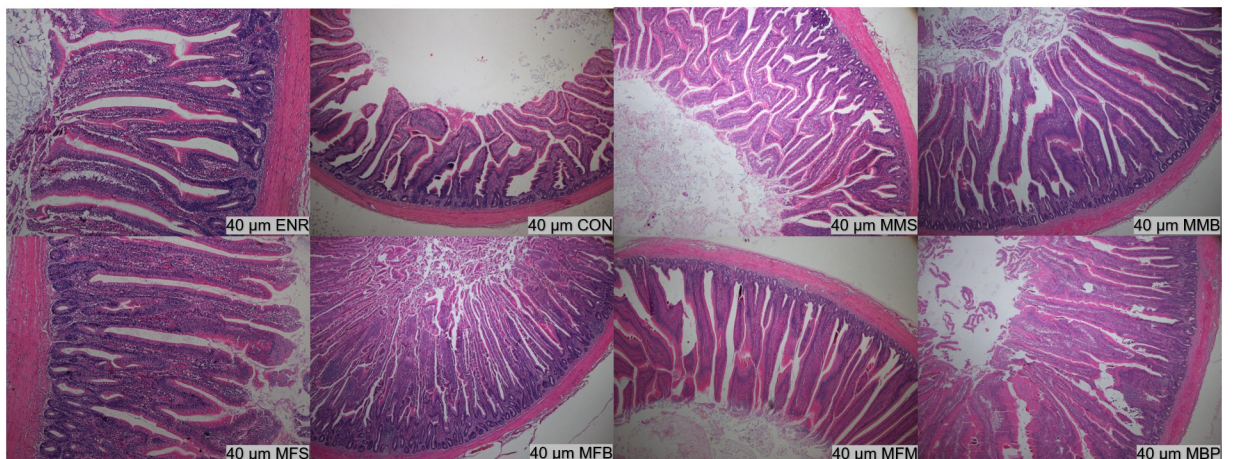


**Fig 3. Histological structure of Hematoxylin and Eosin (H&E) staining jejunum at 42 day of age.** CON, control diet; ENR; CON + 100 mg/kg ENR, MMS; CON + 2000 mg/kg MOS + 300 mg/kg MAN + 1500 mg/kg SB, MMB; CON + 2000 mg/kg MOS + 300 mg/kg MAN + 500 mg/kg BS, MFS; CON + 2000 mg/kg MOS + 9000 mg/kg FOS + 1500 mg/kg SB, MFB; CON + 2000 mg/kg MOS + 9000 mg/kg FOS + 500 mg/kg BS, MFM; CON + 2000 mg/kg MOS + 9000 mg/kg FOS + 300 mg/kg MAN, MBP; CON + 2000 mg/kg MOS + 500 mg/kg BS + 37 mg/kg PT. SB; Sodium butyrate, FOS; Fructose oligosaccharide, PT; Phytase, BS; Bacillus subtilis, MOS; Mannose oligosaccharide, MAN; Mannanase.
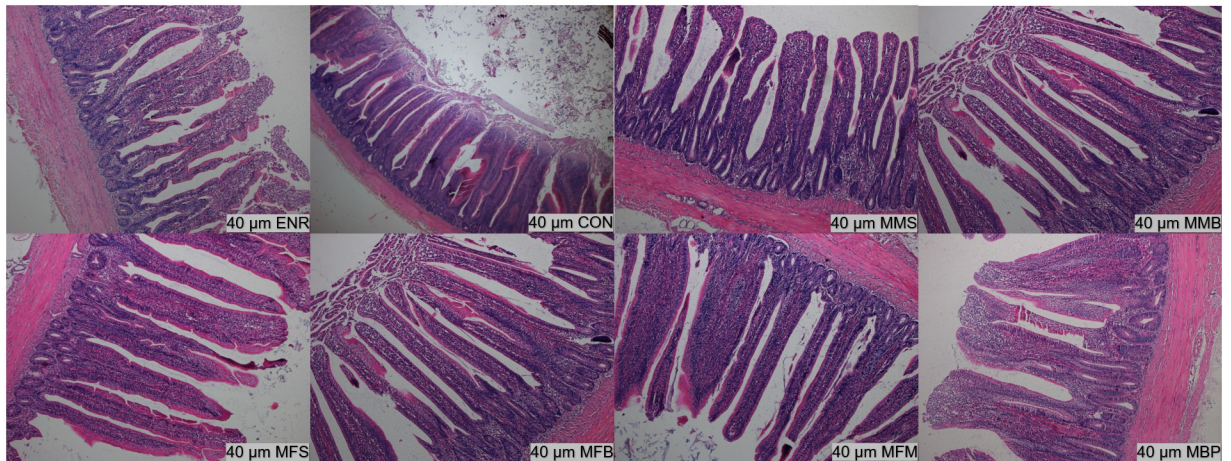
**Fig 4. Histological structure of Hematoxylin and Eosin (H&E) staining ileum at 42 day of age.** CON, control diet; ENR; CON + 100 mg/kg ENR, MMS; CON + 2000 mg/kg MOS + 300 mg/kg MAN + 1500 mg/kg SB, MMB; CON + 2000 mg/kg MOS + 300 mg/kg MAN + 500 mg/kg BS, MFS; CON + 2000 mg/kg MOS + 9000 mg/kg FOS + 1500 mg/kg SB, MFB; CON + 2000 mg/kg MOS + 9000 mg/kg FOS + 500 mg/kg BS, MFM; CON + 2000 mg/kg MOS + 9000 mg/kg FOS + 300 mg/kg MAN, MBP; CON + 2000 mg/kg MOS + 500 mg/kg BS + 37 mg/kg PT. SB; Sodium butyrate, FOS; Fructose oligosaccharide, PT; Phytase, BS; Bacillus subtilis, MOS; Mannose oligosaccharide, MAN; Mannanase.

and ENR groups, the relative abundance of *Sellimonas* in the other groups was increased and that of *Pseudochrobactrum* was decreased at 21 d. Compared with that in the CON group, the relative abundance of *UCG-013* in *Ruminococcaceae* was increased at 42 d in the other groups.

## Diversity of cecal microbiota

**Alpha diversity.** The alpha diversity indexes were calculated based on the OTUs using the Shannon, Simpson, and Chao1 methods. No significant differences in the alpha diversity indexes (including OTU, Shannon, Simpson, and Chao1) of the cecal microbiota in broilers were observed at 21 and 42 d.

**Beta diversity.** Beta diversity analysis was performed to compare the degree of similarity among different samples with respect to species. Beta diversity was assessed by PCoA using the weighted UniFrac distance method. Fig 7A and 7B show the PCoA results of the variation among the eight groups at 21 and 42 d; no significant difference in species diversity was observed. Linear discriminant analysis effect size (LEfSe) analysis was used to determine biomarkers with significant differences in expression among the different treatments. Fig 7C and 7D show the species with significant differences among the eight groups at 21 and 42 d with LDA scores > 4. *Ruminiclostridium* at 21d as well as *Tannerellaceae* and *Parabacteroides* at 42d showed LDA scores > 4 in the MMS group.

## Discussion

Due to the absence of AGPs, alternate methods need to be developed to ensure feed efficiency and broiler health, and now NAGPCs that combine the same kinds of NAGPs in broilers may limit the beneficial effect of synergy. The aim of the current experiment was to evaluate the effects of six NAGPCs with different types of NAGPs on growth performance, nutrient utilization, digestive enzyme activity, intestinal morphology, and cecal microflora of broilers so as to find a better alternative method to ensure feed efficiency and the broiler health. The beneficial effects of NAGPCs on broiler performance reported in this study may be due to the different beneficial mechanisms of each additive and the known synergistic effects of probiotics and
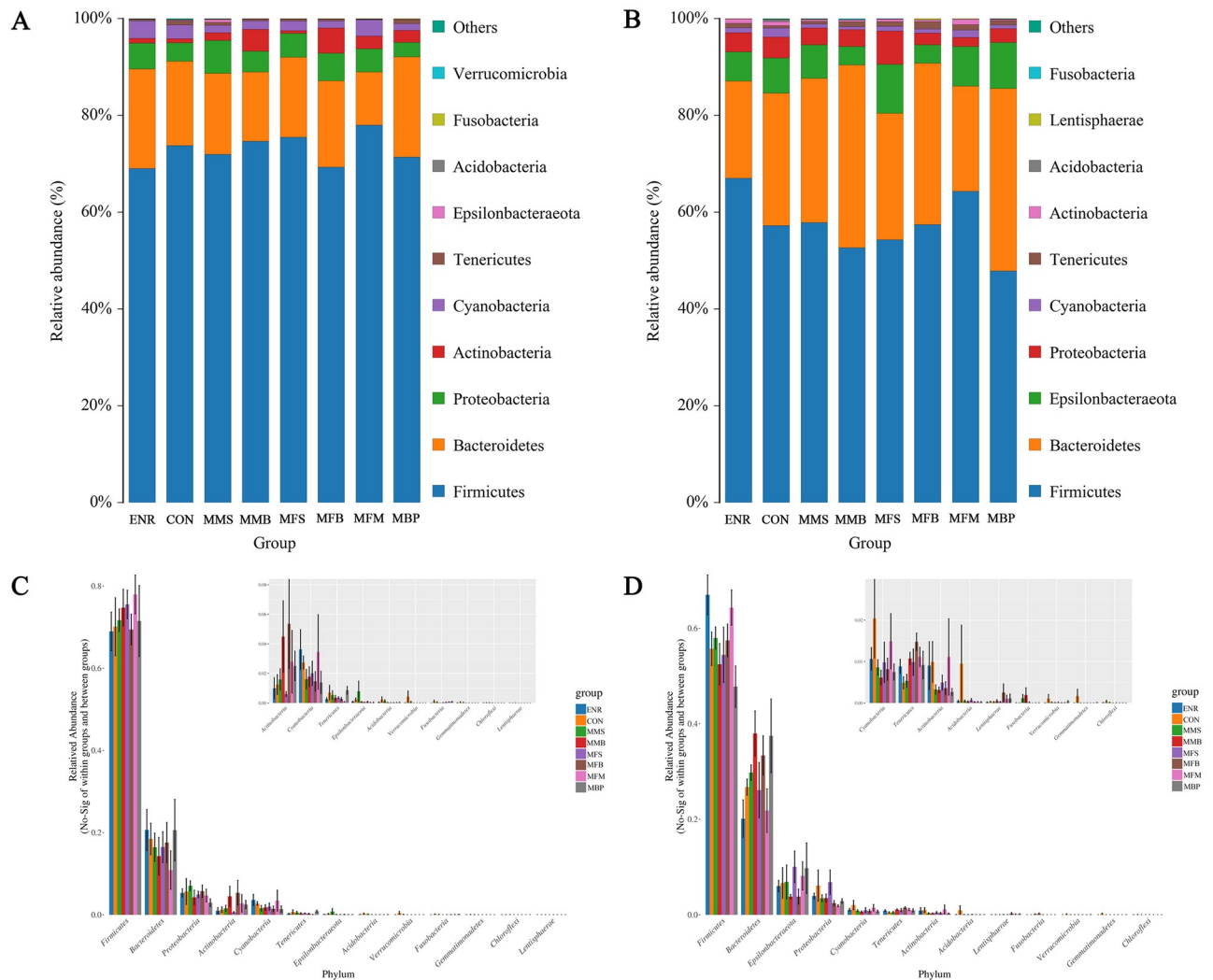
**Fig 5. Different taxa and significantly different taxa between different groups by Anova analysis at the phylum level at 21 and 42 days old.** (A) and (C) 21d. (B) and (D) 42d. CON, control diet; ENR; CON + 100 mg/kg ENR, MMS; CON + 2000 mg/kg MOS + 300 mg/kg MAN + 1500 mg/kg SB, MMB; CON + 2000 mg/kg MOS + 300 mg/kg MAN + 500 mg/kg BS, MFS; CON + 2000 mg/kg MOS + 9000 mg/kg FOS + 1500 mg/kg SB, MFB; CON + 2000 mg/kg MOS + 9000 mg/kg FOS + 500 mg/kg BS, MFM; CON + 2000 mg/kg MOS + 9000 mg/kg FOS + 300 mg/kg MAN, MBP; CON + 2000 mg/kg MOS + 500 mg/kg BS + 37 mg/kg PT. SB; Sodium butyrate, FOS; Fructose oligosaccharide, PT; Phytase, BS; Bacillus subtilis, MOS; Mannose oligosaccharide, MAN; Mannanase.

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prebiotics. The improvement in the performance of broiler chicken fed with probiotics is thought to be due to the maintenance of beneficial microbial populations [22], improvement in feed intake and nutrient digestibility [23, 24], and alteration of bacterial metabolism [25]. However, compared to the use of prebiotics and probiotics alone, their combination significantly improved the growth performance of broilers [26, 27], which may be related to their synergistic mechanism. In addition, it was found that the addition of MAN or PT to the basal diet can effectively improve the growth performance of broilers [28–30], which may be due to the following: (1) the beneficial effect of MAN in reducing the action of mannan;(2) improvement of nutrient absorption through the release of encapsulated nutrients via the breakdown of the cell wall matrix; and (3) a decrease in digesta viscosity [31]. The benefits of supplementing PT have been attributed to effects beyond phosphorus liberation, such as further phytate
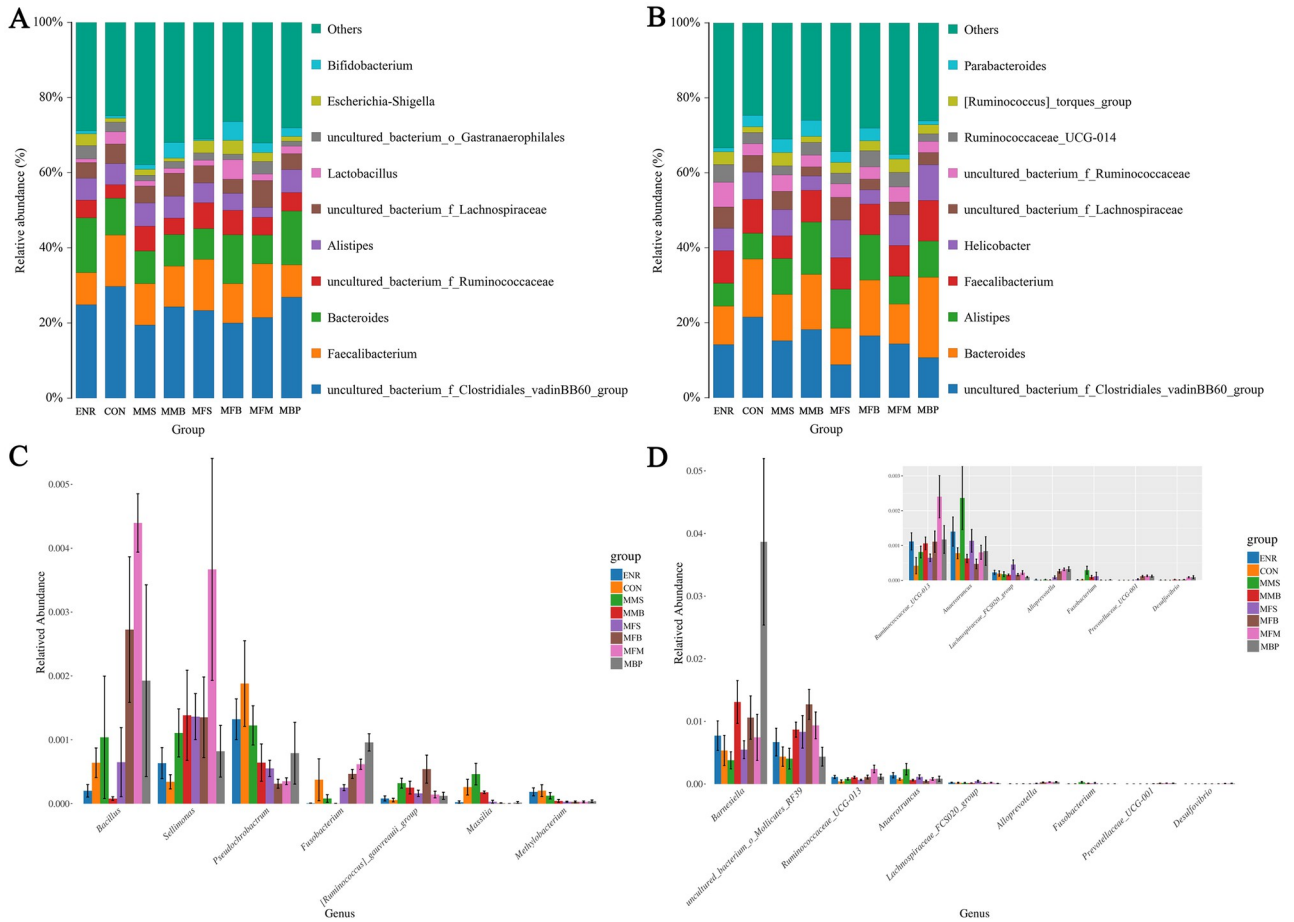
**Fig 6. Different taxa and significantly different taxa between different groups by Anova analysis at the genus level at 21 and 42 days old.** (A) and (C) 21d. (B) and (D) 42d. CON, control diet; ENR; CON + 100 mg/kg ENR, MMS; CON + 2000 mg/kg MOS + 300 mg/kg MAN + 1500 mg/kg SB, MMB; CON + 2000 mg/kg MOS + 300 mg/kg MAN + 500 mg/kg BS, MFS; CON + 2000 mg/kg MOS + 9000 mg/kg FOS + 1500 mg/kg SB, MFB; CON + 2000 mg/kg MOS + 9000 mg/kg FOS + 500 mg/kg BS, MFM; CON + 2000 mg/kg MOS + 9000 mg/kg FOS + 300 mg/kg MAN, MBP; CON + 2000 mg/kg MOS + 500 mg/kg BS + 37 mg/kg PT. SB; Sodium butyrate, FOS; Fructose oligosaccharide, PT; Phytase, BS, Bacillus subtilis, MOS; Mannose oligosaccharide, MAN; Mannanase.

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degradation [32], increased nutrient digestibility [33], and restoration of enzyme functions [34]. Finally, dietary SB supplementation showed a decreasing trend of pH in the duodenum and jejunum; the decreasing pH in the small intestine may have minimized the pathogen load and improved digestibility [35], which may explain the increase in growth performance.

In this study, broilers fed diets supplemented with NAGPCs significantly increased the utilization of DM, OM, CP, and CF at 21 and 42 d as well as the utilization of CA and P at 42 d. The synergistic and complementary effects of additives observed in this study may be attributed to the different beneficial mechanisms of action of each additive [36]. Previous studies have reported that dietary supplementation of MAN [37, 38] and PT [39] results in improved nutritional digestibility in broilers, possibly because of the beneficial effect of MAN in reducing the action of mannan, improving nutrient absorption by the release of encapsulated nutrients through breakdown of the cell wall matrix, and decreasing digesta viscosity [27]. The benefits of PT supplementation have been attributed to effects of further phytate degradation [32] and restoration of enzyme functions [34]. Furthermore, prebiotics may disrupt intestinal
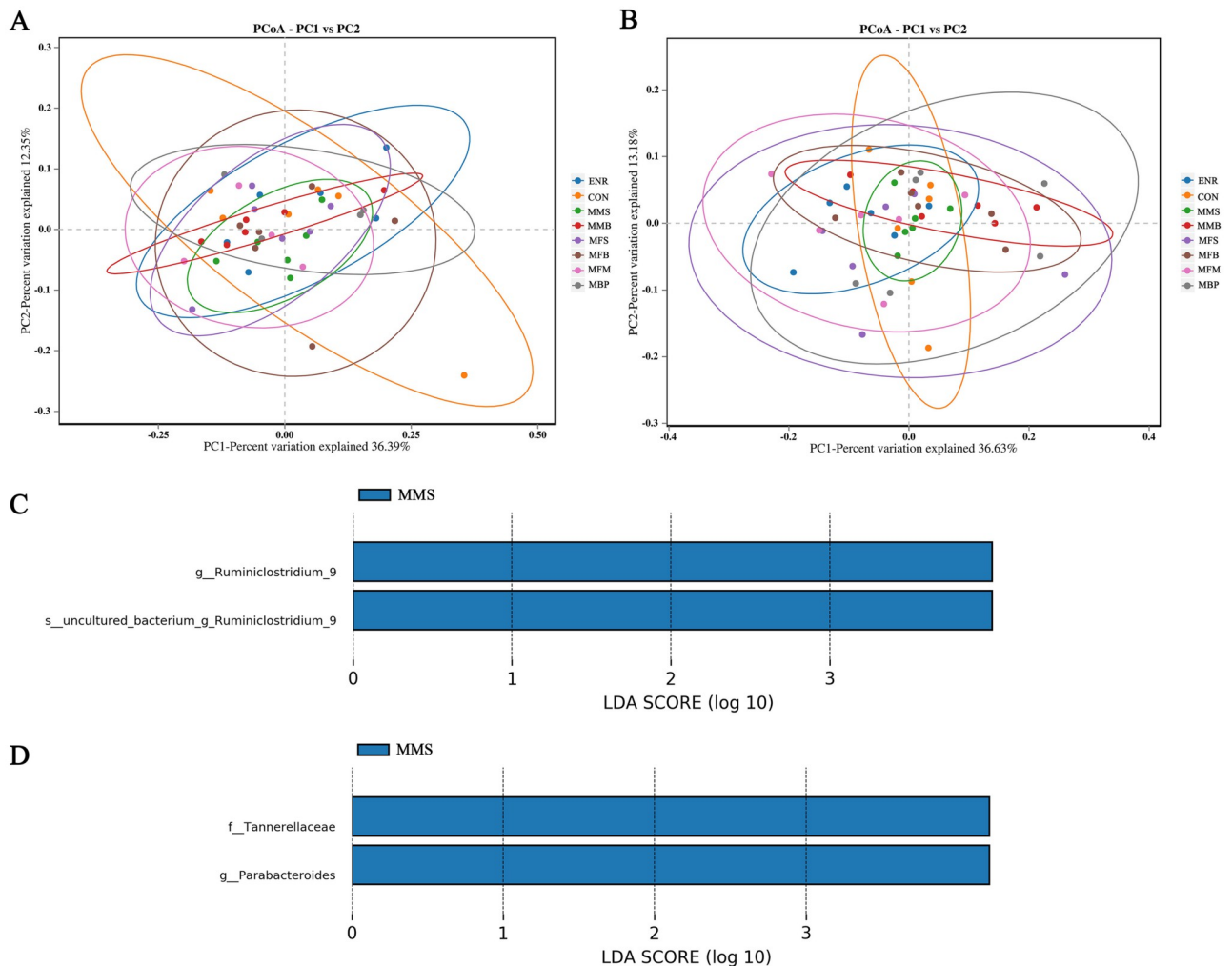
**Fig 7. Beta diversity and Linear discriminant analysis effect size analysis of the microbiome residing in the cecal chyme of broilers at 21 and 42 days old.** (A) PCoA plot at 21d. (B) PCoA plot at 42d. (C) LDA distribution histogram at 21d (LDA scores > 4). (C) LDA distribution histogram at 42d (LDA scores > 4). CON, control diet; ENR; CON + 100 mg/kg ENR, MMS; CON + 2000 mg/kg MOS + 300 mg/kg MAN + 1500 mg/kg SB, MMB; CON + 2000 mg/kg MOS + 300 mg/kg MAN + 500 mg/kg BS, MFS; CON + 2000 mg/kg MOS + 9000 mg/kg FOS + 1500 mg/kg SB, MFB; CON + 2000 mg/kg MOS + 9000 mg/kg FOS + 500 mg/kg BS, MFM; CON + 2000 mg/kg MOS + 9000 mg/kg FOS + 300 mg/kg MAN, MBP; CON + 2000 mg/kg MOS + 500 mg/kg BS + 37 mg/kg PT. SB; Sodium butyrate, FOS; Fructose oligosaccharide, PT; Phytase, BS; Bacillus subtilis, MOS; Mannose oligosaccharide, MAN; Mannanase.

pathogen colonization and improve the intestinal environment. This is the main reason for improved nutrient utilization [40]. SB has a similar effect, as it helps broilers to reduce the number of pathogens in the digestive tract and regulate intestinal microflora [41, 42]. Studies have shown that adding a combination of prebiotics and butyric acid to the diet of broilers can better control *Salmonella typhimurium* abundance and promote growth compared with addition of prebiotics or butyric acid alone. This could be attributed to the synergistic effect of prebiotics and butyric acid [36]. BS can increase the length of intestinal villi and crypt depth, thus increasing the digestibility of nutrients [43]. Besides, the probiotics use the prebiotics as a food source, which enables them to survive for a longer period of time inside the human digestive system than would otherwise be possible [44]. Finally, the increase in the nutritional utilization rate of birds treated with NAGPCs may be related to the increase in duodenal digestive enzyme activity.

In this study, broilers fed diets supplemented with NAGPCs showed higher activity of trypsin, lipase, and amylase. Some species of pathogenic bacteria, such as *Escherichia coli* [45] and *Clostridium* [46], have been shown to inhibit digestive enzyme secretion by damaging the villi and microvilli in the intestinal mucosa. Organic acids can lower the pH of the chyme, which minimizes the pathogen load and enhances the digestibility of protein by improving pepsin activity [47]. Moreover, they can enhance the production of pancreatic juice containing various zymogens (trypsinogen, chymotrypsinogens A and B, and procarboxypeptidases A and B), leading to improved digestive enzyme activity [48]. Prebiotics are non-digestible carbohydrates that selectively stimulate *Bifidobacterium* and *Lactobacillus* growth [49]; furthermore, the effect of MAN in reducing intestinal viscosity has been suggested to prepare a suitable environment for *Lactobacillus* growth [50]. *Bifidobacterium* and *Lactobacillus* colonizing the intestine have been reported to deliver enzymes [51], explaining why prebiotics and MAN increase the activity of digestive enzymes. Regarding BS, their presence along intestinal sections might stimulate the production of endogenous enzymes by broilers [52]. Furthermore, BS itself can produce protease and amylase [53]. Research has shown that phytate can chelate with the cofactors required for optimum enzyme activity to reduce the activity of digestive enzymes [54] whereas PT can promote the degradation of phytate, thus increasing the activity of digestive enzymes [32]. Finally, the observed synergistic and complementary effect of the additives used in this study can be attributed to the different beneficial mechanisms of action of each NAGP.

Villi and crypts are the two key components of the small intestine, and their shape offers an indication of absorptive ability [2]. Increasing villus height provides an increased surface area for the absorption of available nutrients [55]. The villus crypt is considered the villus factory, and deeper crypts indicate rapid tissue turnover to permit villus renewal as needed in response to normal sloughing or inflammation from pathogens or their toxins and high tissue demands [56, 57]. Intestinal epithelial cells originating in the crypt migrate along the villus surface upward to the villus tip and are extruded into the intestinal lumen within 48 to 96 h [58, 59]. Shortening of the villi and deepening crypts may lead to poor nutrient absorption, increased secretion in the gastrointestinal tract, and lower performance [60]. In contrast, increased villus height and villus height:crypt depth ratio are directly correlated with increased epithelial cell turnover [61], and longer villi are associated with activated cell mitosis [62]. The present results indicated that supplementing NAGPC in broiler diets could improve the villus height and villus height/crypt depth as well as decrease crypt depth in the jejunum and ileum. Previous research indicates that the addition of synbiotics or probiotics can increase the turnover rate of epithelial cells, improve intestinal tissue structure, and increase the ratio of villus height to crypt depth in the ileum [27]. Further, addition of PT to the diet may decline the growth of intestinal pathogenic bacteria by possibly decreasing the quantity of available substrates for their metabolization [63]; this mechanism also reduces the pathogen-induced damage to the intestinal mucosa [64]. MAN can reduce digesta viscosity by degrading β-mannan, one of the major soluble non-starch polysaccharides in the diet, and thus ameliorate structural damage to the absorptive architecture [65]. Finally, SB can be used by intestinal cells to stimulate intestinal development [66] and significantly improve the morphology of the jejunum and ileum [67].

The intestinal microbiota has a significant impact on the control of host homeostasis, organ development, metabolic processes, and immunological response [68, 69]. In this study, the improvement of cecal microflora by NAGPC supplementation may be attributed to the synergistic effect of different NAGPs. Prebiotics are reported to have a beneficial effect on the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the intestine or gut [40]. BS can compete with pathogens, balance intestinal microbiota [70]. Organic acids can reduce cecal pH and inhibit the growth of pathogenic bacteria [71] as well

as adjust the acidic environment to facilitate *Lactobacillus* survival [72]. The correlation between increased digesta viscosity and increased intestinal concentrations of pathogenic bacteria has previously been reported in broilers [73]; MAN can reduce the viscosity of digestive juice, thus promoting the growth of beneficial microflora [74]. Differences in dietary phosphorus content can also affect the cecal microbial diversity of broilers [63]; phytase can promote the degradation of phytate and release P and other nutrients [75], which may improve the cecal microflora.

In this study, we analyzed the changes in cecal microbial composition at the phylum and genus level. At the phylum level, *Firmicutes* and *Bacteroides* were the dominant bacterial groups, accounting for more than 80% of the total microbial communities detected in this study. *Firmicutes* and *Bacteroides* were shown to be the major phyla of the cecal population in 21 and 42 d broilers in our experiment. This is consistent with prior research, as these bacteria are known to play a role in energy generation and metabolism [76]. The dominating groups of the chosen chickens may alter according to changes in age, breed, and area. According to the current research, *Firmicutes* and *Bacteroides* are the major phyla of the cecal community in 42 d broilers supplemented with dietary BS [77], prebiotics [78], organic acids [79], PT [80], and MAN [50]. Further, *Firmicutes* and *Bacteroides* are involved in carbon metabolism [81] and fat deposition [82] whereas *Proteobacteria* comprises zoonotic disease-causing bacteria such as *Escherichia coli*, *Salmonella*, *Campylobacter*, and other well-known pathogens [83]. The results of our study showed that compared with those in the ENR and CON groups, the abundance of *Firmicutes* at 21 and the abundance of *Bacteroides* at 42 d was increased in the MMS, MMB, and MBP and the abundance of *Proteobacteria* was decreased at 21 and 42 d in the MMB, MFB, and MBP groups.

At the genus level, *Clostridiales*, *Bacteroides*, and *Feacalibacterium* were the dominant genera of the cecal community in 21d broilers in our trial, *Clostridiales*, *Bacteroides*, and *Alistipes* were the dominant genera of the cecal community in 42 d broilers in our trial. *Feacalibacterium* is a butyric acid-generating bacterium found in chicken cecum [84]. *Feacalibacterium* may supply energy to the body and alleviate inflammation; its presence thus signals the host intestinal health [85, 86]. *Alistipes* exert anti-inflammatory properties and may protect against some illnesses [87]. *Clostridiales* are involved in the metabolism of intestinal proteins [88]. *Pseudochrobactrum*, is a potential hazard as a pathogen [89]. The results of our study showed that compared with those in the ENR and CON, NAGPCs addition to the diet could decrease the abundance of *Pseudochrobactrum* at 21d. *Sellimonas* is a potential biomarker of intestinal homeostasis [90], the results show that compared with that in ENR and CON, diets supplemented with NAGPCs could increase the abundance of *Sellimonas* at 21d.

The diversity of intestinal microflora is critical for maintaining the gastrointestinal equilibrium and is helpful to the host health [69]. In our study, dietary treatments failed to modify the overall diversity of cecal microbiota at 21 and 42 d. These results were consistent with previous reports. Current research indicates that supplementation with BS [91], FOS, MOS [40], SB [92], MAN [93], and PT [94] has no effect on the diversity and community structure of cecal microbiota in broilers. In fact, aging has a greater impact on microbiota than that of therapy [95]. *Ruminiclostridium* at 21d and *Tannerellaceae* and *Parabacteroides* at 42d with LDA scores > 4 were observed in the MMS group. *Ruminiclostridium*, as a beneficial bacterium, can secrete cellulase and related plant cell wall-degrading enzymes, thereby degrading cellulose in the feed and releasing nutrients [96]. *Tannerellaceae* can secrete butyric acid and propionic acid to regulate cecal pH [97]. *Parabacteroides* have the physiological characteristics of carbohydrate metabolism and secrete short chain fatty acids [98]. This may be caused by the synergistic effect of MAN, MOS, and SB.

## Conclusion

As legislation and public demand for "antibiotic-free" poultry increase the pressure to abandon the use of AGPs, other techniques to stimulate broiler chicken development are being explored. The present research compared six NAGPCs to evaluate their effectiveness as growth promoters and to analyze indicators of broiler health. The results showed that compared with the CON and ENR groups, MMS, MMB, MFS, MFB, MFM, and MBP groups showed significantly improved growth performance. Nutrient utilization, duodenal digestive enzyme activity, and ileal and jejunal histology are promising biomarkers for explaining these growth performance effects. In conclusion, it was found that the six NAGPCs evaluated in this study may enhance the growth performance of broilers that are fed corn-soybean diets that are adequate in their nutritional profiles. This suggests that these NAGPCs may replace AGP in broiler diets. However, the specific mechanisms of action need identified for successfully replacing antibiotic growth promoters. Overall, ideal combinations of different alternatives are likely to be the key to increasing broiler performance and preserving productivity.

## Supporting information

**S1 File. The screening process for the in vitro digestion tests.**
(PDF)

## Author Contributions

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## References

1. Palumbi SR. Humans as the World's Greatest Evolutionary Force the Pace of Human-Induced Evolution. Science. 2001; 293(5536):1786–1790. https://doi.org/10.1126/science.293.5536.1786

2. Heydarian M, Ebrahimnezhad Y, Meimandipour A, Hosseini SA, Banabazi MH. Effects of Dietary Inclusion of the Encapsulated Thyme and Oregano Essential Oils Mixture and Probiotic on Growth Performance, Immune Response and Intestinal Morphology of Broiler Chickens. Poultry Science Journal. 2020; 8(1):17–25. https://doi.org/10.22069/PSJ.2020.17101.1497

3. Zhao C, Ge B, De VJ, Sudler R, Yeh E, Zhao S, et al. Prevalence of Campylobacter spp., Escherichia coli, and Salmonella serovars in retail chicken, turkey, pork, and beef from the Greater Washington, D.

C., area. Applied and Environmental Microbiology. 2001; 67(12):5431–5436. https://doi.org/10.1128/AEM.67.12.5431-5436.2001 PMID: 11722889

4. Diarra MS, Malouin F. Antibiotics in Canadian poultry productions and anticipated alternatives. FRONTIERS IN MICROBIOLOGY. 2014; 17(5):282. https://doi.org/10.3389/fmicb.2014.00282 PMID: 24987390

5. Attia YA, El-Hanoun AM, Bovera F, Monastra G, El-Tahawy WS, Habiba HI. Growth performance, carcass quality, biochemical and haematological traits and immune response of growing rabbits as affected by different growth promoters. Journal of Animal Physiology & Animal Nutrition. 2014; 98 (1):128–139. https://doi.org/10.1111/jpn.12056 PMID: 23419029.

6. Abd El-Hack ME, El-Saadony MT, Shafi ME, Qattan SYA, Batiha GE, Khafaga AF, et al. Probiotics in poultry feed: A comprehensive review. Journal of Animal Physiology & Animal Nutrition. 2020; 104 (6):1835–1850. https://doi.org/10.1111/jpn.13454 PMID: 32996177

7. Ravindran V, Son JH. Feed enzyme technology: present status and future developments. Recent patents on food, nutrition & agriculture. 2011; 3(2):102–109. https://doi.org/10.2174/2212798411103020102 PMID: 21428871

8. Morgan NK. Managing gut health without reliance on antimicrobials in poultry. Animal Production Science. 2017; 57(11):2270–2279. https://doi.org/10.1071/AN17288

9. Lai Y, Zhang Y, Yang H, Qu X, Yu C. Effect of Sodium Butyrate on Restoration of Colonic Mucosa in Rats with TNBS-induced Colitis. Chinese Journal of Gastroenterology 2011; 16(7): 395–399. https://doi.org/10.3969/j.issn.1008-7125.2011.07.003

10. Smirnov VV, Sorokulova IB, Pinchuk IV. Bacteria of Bacillus species—prospective source for biologically active substances. Mikrobiolohichnyi. Mikrobiolohichnyi zhurnal. 2001; 63(1):72–79. PMID: 11392775

11. Barre A, Bourne Y, Van Damme E, Rougé P. Overview of the Structure–Function Relationships of Mannose-Specific Lectins from Plants, Algae and Fungi. International Journal of Molecular Sciences. 2019; 20(2):254. https://doi.org/10.3390/ijms20020254 PMID: 30634645

12. Upadhaya SD, Park JW, Lee JH, Kim IH. Efficacy of β-mannanase supplementation to corn-soya bean meal-based diets on growth performance, nutrient digestibility, blood urea nitrogen, faecal coliform and lactic acid bacteria and faecal noxious gas emission in growing pigs. Archives of Animal Nutrition 2016; 70(1): 33–43. https://doi.org/10.1080/1745039X.2015.1117697 PMID: 26635142

13. Wodzinski RJ, Ullah AH. Phytase. Advances in Applied Microbiology. 1996; 42(42):263–302. https://doi.org/10.1016/s0065-2164(08)70375-7 PMID: 8865587

14. Al-Kassi AG, Mohssen MA. Comparative Study Between Single Organic Acid Effect and Synergistic Organic Acid Effect on Broiler Performance. Pakistan Journal of Nutrition. 2009; 8(6):1680–5194 https://doi.org/10.3923/pjn.2009.896.899

15. Olukosi OA, Beeson LA, Englyst K, Romero LF. Effects of exogenous proteases without or with NSP-hydrolysing enzymes on nutrient digestibility and disappearance of non-starch polysaccharides in broiler chickens. Poultry Science. 2015; 94(11):2662–2669. https://doi.org/10.3382/ps/pev260

16. Graham H, Löwgren W, Aman P. An in vitro method for studying digestion in the pig. 2. Comparison with in vivo ileal and faecal digestibilities. Brith Journal Nutrition. 1989; 61(3):689–98. https://doi.org/10.1079/bjn19890155 PMID: 2547431

17. Pedersen NR, Azem E, Broz J, Guggenbuhl P, Le DM, Fojan P, et al. The degradation of arabinoxylan-rich cell walls in digesta obtained from piglets fed wheat-based diets varies depending on digesta collection site, type of cereal, and source of exogenous xylanase. Journal of Animal Science. 2012; 90 (Suppl4):149–151. https://doi.org/10.2527/jas.53832 PMID: 23365312

18. NATIONAL RC. Nutrient requirement of poultry. National Academy Press; 1994.

19. Gaithersburg (Maryland), USA: Association of Official Analytical Chemists. AOAC. Official Methods of Analysis of AOAC international. 2006;18.

20. AOAC Int., Washington DC. AOAC. Official Methods of Analysis. 1990;15.

21. AOAC Int., Gaithersburg MD. AOAC. Official Methods of Analysis. 2000;18.

22. Teo AY, Tan HM. Evaluation of the performance and intestinal gut microflora of broilers fed on corn-soy diets supplemented with Bacillus subtilis PB6 (CloSTAT). Journal of Applied Poultry Research. 2007; 16(3):296–303. https://doi.org/10.1093/japr/16.3.296

23. Attia YA, Allakany HF, Abd Al-Hamid AE, Al-Saffar AA, Hassan RA, Mohamed NA. Capability of different non-nutritive feed additives on improving productive and physiological traits of broiler chicks fed diets with or without aflatoxin during the first 3 weeks of life. Journal of Animal Physiology and Animal Nutrition. 2013; 97(4):754–772. https://doi.org/10.1111/j.1439-0396.2012.01317.x PMID: 23050696

24. Attia YA, Zeweil HS, Alsaffar AA, El-Shafy AS. Effect of non-antibiotic feed additives as an alternative to flavomycin on broiler chickens production. Archiv Für Geflügelkunde. 2011; 75(1):40–48.

**25.** Jin LZ, Ho YW, Abdullah N, Jalaludin S. Probiotics in poultry: Modes of action. World's Poultry Science. 1997; 53(4):352–368. https://doi.org/10.1079/WPS19970028

**26.** Wang X, Farnell YZ, Peebles ED, Kiess AS, Wamsley KG, Zhai W. Effects of prebiotics, probiotics, and their combination on growth performance, small intestine morphology, and resident Lactobacillus of male broilers. Poultry Science. 2016; 95(6):1332–1340. https://doi.org/10.3382/ps/pew030 PMID: 26944975

**27.** Awad WA, Ghareeb K, Abdel-Raheem S, Böhm J. Effects of dietary inclusion of probiotic and synbiotic on growth performance, organ weights, and intestinal histomorphology of broiler chickens. Poultry Science. 2009; 88(1):49–56. https://doi.org/10.3382/ps.2008-00244 PMID: 19096056

**28.** Ferreira HC, Hannas MI, Albino LFT, Rostagno HS, Neme R, Faria BD, et al. Effect of the addition of β-mannanase on the performance, metabolizable energy, amino acid digestibility coefficients, and immune functions of broilers fed different nutritional levels. Poultry Science. 2016; 95(8):1848–1857. https://doi.org/10.3382/ps/pew076 PMID: 27038422

**29.** Kiarie EG, Steelman S, Martinez M, Livingston K. Significance of single β-mannanase supplementation on performance and energy utilization in broiler chickens, laying hens, turkeys, sows, and nursery-finish pigs: a meta-analysis and systematic review. Translational Animal Science. 2021; 5(4):txab160. https://doi.org/10.1093/tas/txab160 PMID: 34888489

**30.** Kriseldi R, Walk CL, Bedford MR, Dozier WA. Inositol and gradient phytase supplementation in broiler diets during a 6-week production period: 1. effects on growth performance and meat yield. Poultry Science. 2021; 100(2):964–972. https://doi.org/10.1016/j.psj.2020.11.051 PMID: 33518150

**31.** Mehri M, Adibmoradi M, Samie A, Shivazad M. Effects of β-Mannanase on broiler performance, gut morphology and immune system. African Journal of Immunology Research. 2010; 4(3):211–217.

**32.** Zeller E, Schollenberger M, Witzig M, Shastak Y, Kuhn I, Hoelzle LE, et al. Interaction between supplemented mineral phosphorus and phytase on phytate hydrolysis and inositol phosphates in the small intestine of broilers. Poultry Science. 2015; 94(5):1018–1029. https://doi.org/10.3382/ps/pev087 PMID: 25810408

**33.** Gehring CK, Bedford MR, Dozier WA. Extra phosphoric effects of phytase with and without xylanase in corn-soybean meal-based diets fed to broilers. Poultry Science. 2013; 92(4):979–991. https://doi.org/10.3382/ps.2012-02769 PMID: 23472022

**34.** Liu N, Ru YJ, Li FD, Cowieson AJ. Effect of diet containing phytate and phytase on the activity and messenger ribonucleic acid expression of carbohydrase and transporter in chickens. Journal Animal Science. 2008; 86(12):3432–3439. https://doi.org/10.2527/jas.2008-1234 PMID: 18708594

**35.** Hassan H, Mohamed M, Youssef AW, Hassan ER. Effect of using organic acids to substitute antibiotic growth promoters on performance and intestinal microflora of broilers. Asian-Australasian Journal of Animal Sciences. 2010; 23(10):1348–1353. https://doi.org/10.5713/ajas.2010.10085

**36.** Jazi V, Foroozandeh AD, Toghyani M, Dastar B, Rezaie KR, Toghyani M. Effects of Pediococcus acidilactici, mannan-oligosaccharide, butyric acid and their combination on growth performance and intestinal health in young broiler chickens challenged with Salmonella Typhimurium. Poultry science. 2018; 97(6): 2034–2043. https://doi.org/10.3382/ps/pey035 PMID: 29514269

**37.** Balasubramanian B, Ingale SL, Park JH, Rathi PC, Shanmugam S, Kim IH. Inclusion of dietary β-mannanase improves performance and ileal digestibility and reduces ileal digesta viscosity of broilers fed corn-soybean meal based diet. Poultry Science. 2018; 97(9):3097–3101. https://doi.org/10.3382/ps/pey157 PMID: 29771358.

**38.** Mussini FJ, Coto CA, Goodgame SD, Lu C, Karimi AJ, Lee JH, et al. Effect of β-Mannanase on Broiler Performance and Dry Matter Output Using Corn-Soybean Meal Based Diets. International Journal of Poultry Science. 2011; 10(10):778–781. https://doi.org/10.3923/ijps.2011.778.781

**39.** Chung TK, Rutherfurd SM, Thomas DV, Moughan PJ. Effect of two microbial phytases on mineral availability and retention and bone mineral density in low-phosphorus diets for broilers. British Poultry Science. 2013; 54(3):362–373. https://doi.org/10.1080/00071668.2013.783902 PMID: 23662985

**40.** Yang GQ, Yin Y, Liu HY, Liu GH. Effects of dietary oligosaccharide supplementation on growth performance, concentrations of the major odor-causing compounds in excreta, and the cecal microflora of broilers. Poultry science. 2016; 95(10):2342–2351. https://doi.org/10.3382/ps/pew124 PMID: 27081199

**41.** Bortoluzzi C, Pedroso AA, Mallo JJ, Puyalto M, Kim WK, Applegate TJ. Sodium butyrate improved performance while modulating the cecal microbiota and regulating the expression of intestinal immune-related genes of broiler chickens. Poultry science. 2017; 96(11):3981–3993. https://doi.org/10.3382/ps/pex218 PMID: 29050425

**42.** Fernandez-Rubio C, Ordonez C, Abad-Gonzalez J, Garcia-Gallego A, Honrubia MP, Mallo JJ, et al. Butyric acid-based feed additives help protect broiler chickens from Salmonella Enteritidis infection. Poultry science. 2009; 88(5):943–948. https://doi.org/10.3382/ps.2008-00484 PMID: 19359681

43. Rivera-Pérez W, Chaves AJ, Barquero-Calvo E. Effect of the use of probiotic Bacillus subtilis (QST 713) as a growth promoter in broilers: an alternative to Bacitracin Methylene Disalicylate. Poultry science. 2021; 100(9):101372. https://doi.org/10.1016/j.psj.2021.101372 PMID: 34364120

44. Sekhon BS, Jairath S. Prebiotics, probiotics and synbiotics: An overview. South Journal of Pharmaceutical Education and Research. 2010; 1(2):13–36.

45. Zhang L, Zhang L, Zeng X, Zhou L, Cao G, Yang C. Effects of dietary supplementation of probiotic, Clostridium butyricum, on growth performance, immune response, intestinal barrier function, and digestive enzyme activity in broiler chickens challenged with Escherichia coli K88. 2016; 7(1):9. https://doi.org/10.1186/s40104-016-0061-4 PMID: 26819705

46. Mitsch P, Zitterl-Eglseer K, Kohler B, Gabler C, Losa R, Zimpernik I. The effect of two different blends of essential oil components on the proliferation of Clostridium perfringens in the intestines of broiler chickens. Poultry Science. 2004; 83(4):669–675. https://doi.org/10.1093/ps/83.4.669 PMID: 15109065

47. Khan RU, Naz S, Raziq F, Qudratullah Q, Khan NA, Laudadio V, et al. Prospects of organic acids as safe alternative to antibiotics in broiler chickens diet. Environmental science and pollution research international. 2022; 29(22): 32594–32604. https://doi.org/10.1007/s11356-022-19241-8 PMID: 35195862

48. Adil S, Banday T, Bhatm GA, Mir MS, Rehman M. Effect of dietary supplementation of organic acids on performance, intestinal histomorphology, and serum biochemistry of broiler chicken. Veterinary Medicine International. 2010; 2010:479485. https://doi.org/10.4061/2010/479485 PMID: 20613998

49. Ricke SC, Lee SI, Kim SA, Park SH, Shi Z. Prebiotics and the poultry gastrointestinal tract microbiome. Poultry science. 2020; 99(2):670–677. https://doi.org/10.1016/j.psj.2019.12.018 PMID: 32029153

50. Mohammadigheisar M, Shouldice VL, Balasubramanian B, Kim IH. Effect of dietary supplementation of β-mannanase on growth performance, carcass characteristics, excreta microflora, blood constituents, and nutrient ileal digestibility in broiler chickens. Animal bioscience. 2021; 34(8):1342–1349. https://doi.org/10.5713/ab.20.0355

51. Sissons JW. Potential of probiotic organisms to prevent diarrhea and promote digestion in farm animals: a review. Journal of the Science of Food and Agriculture. 1989; 49(1):1–13. https://doi.org/10.1002/jsfa.2740490102

52. Wang Y, Heng C, Zhou X, Cao G, Jiang L, Wang J, et al. Supplemental Bacillus subtilis DSM 29784 and enzymes, alone or in combination, as alternatives for antibiotics to improve growth performance, digestive enzyme activity, anti-oxidative status, immune response and the intestinal barrier of broiler chickens. The British journal of nutrition. 2021; 125(5):494–507. https://doi.org/10.1017/S0007114520002755 PMID: 32693847

53. Sun H, Tang JW, Yao XH, Wu YF, Wang X, Feng J. Effects of dietary inclusion of fermented cottonseed meal on growth, cecal microbial population, small intestinal morphology, and digestive enzyme activity of broilers. Tropical Animal Health & Production. 2013; 45(4):987–993. https://doi.org/10.1007/s11250-012-0322-y PMID: 23224950

54. Katayama T. Effects of dietary myo-inositol or phytic acid on hepatic concentrations of lipids and hepatic activities of lipogenic enzymes in rats fed on corn starch or sucrose. Nutrition Research. 1997; 17(4):721–728. https://doi.org/10.1016/S0271-5317(97)00042-0

55. Caspary WF. Physiology and pathophysiology of intestinal absorption. American Journal of Clinical Nutrition. 1992; 55:299S–308S. https://doi.org/10.1093/ajcn/55.1.299s PMID: 1728844

56. Yason CV, Summers BA, Schat KA. Pathogenesis of rotavirus infection in various age groups of chickens and turkeys: Pathology. American journal of veterinary research. 1987; 48(6):927–938. PMID: 3605809

57. Teng PY, Adhikari R, Llamas-Moya S, Kim WK. Effects of combination of mannan-oligosaccharides and β-glucan on growth performance, intestinal morphology, and immune gene expression in broiler chickens. Poultry Science. 2021; 100(12):101483. https://doi.org/10.1016/j.psj.2021.101483 PMID: 34700101

58. Imondi AR, Bird FH. The turnover of intestinal epithelium in the chick. Poultry Science. 1966; 45(1):142–147. https://doi.org/10.3382/ps.0450142 PMID: 5948767

59. Potten CS. Stem cells in the gastrointestinal epithelium: Numbers, characteristics and death. Philosophical transactions—Royal Society. Biological sciences. 1998; 353(1370):821–830. https://doi.org/10.1098/rstb.1998.0246 PMID: 9684279

60. Xu ZR, Hu CH, Xia MS, Zhan XA, Wang MQ. Effects of dietary fructooligosaccharide on digestive enzyme activities, intestinal microflora and morphology of male broilers. Poultry Science. 2003; 82(6):1030–1036. https://doi.org/10.1093/ps/82.6.1030 PMID: 12817461

61. Fan Y, Croom J, Christensen V, Black B, Bird A, Daniel L, et al. Jejunal glucose uptake and oxygen consumption in turkey poults selected for rapid growth. Poultry science. 1997; 76(12);1738–1745. https://doi.org/10.1093/ps/76.12.1738 PMID: 9438290

62. Samanya M, Yamauchi K. Histological alterations of intestinal villi in chickens fed dried Bacillus subtilis var. natto. Comparative biochemistry and physiology. Part A, Molecular & integrative physiology. 2002; 133(1):95–104. https://doi.org/10.1016/s1095-6433(02)00121-6 PMID: 12160875

63. Borda-Molina D, Seifert J, Camarinha-Silva A. Current perspectives of the chicken gastrointestinal tract and its microbiome. Computational and structural biotechnology journal. 2018; 16:131–139 https://doi.org/10.1016/j.csbj.2018.03.002 PMID: 30026889

64. Amiri MYA, Jafari MA, Irani M. Growth performance, internal organ traits, intestinal morphology, and microbial population of broiler chickens fed quinoa seed-based diets with phytase or protease supplements and their combination. Tropical animal health and production. 2021; 53(6):535. https://doi.org/10.1007/s11250-021-02980-0 PMID: 34743230

65. Jang JC, Kim KH, Jang YD, Kim YY. Effects of Dietary β-Mannanase Supplementation on Growth Performance, Apparent Total Tract Digestibility, Intestinal Integrity, and Immune Responses in Weaning Pigs. Animals: an open access journal from MDPI. 2020; 10(4):703. https://doi.org/10.3390/ani10040703 PMID: 32316523

66. Ma Y, Wang W, Zhang H, Wang J, Zhang W, Gao J, et al. Supplemental Bacillus subtilis DSM 32315 manipulates intestinal structure and microbial composition in broiler chickens. Scientific reports. 2018; 8(1):15358. https://doi.org/10.1038/s41598-018-33762-8 PMID: 30337568

67. Sikandar A, Zaneb H, Younus M, Masood S, Aslam A, Khattak F, et al. Effect of sodium butyrate on performance, immune status, microarchitecture of small intestinal mucosa and lymphoid organs in broiler chickens. Asian-Australasian Journal of Animal Sciences. 2017; 30(5):90–99 https://doi.org/10.5713/ajas.16.0824 PMID: 28111438

68. Tremaroli V, Backhed F. Functional interactions between the gut microbiota and host metabolism. Nature. 2012; 489(7415):242–249 https://doi.org/10.1038/nature11552 PMID: 22972297

69. Zhang L, Wu W, Yuan KJ, Xie J, Zhang H. Spatial Heterogeneity and Co-occurrence of Mucosal and Luminal Microbiome across Swine Intestinal Tract. Frontiers in microbiology. 2018; 9:48. https://doi.org/10.3389/fmicb.2018.00048

70. Guo MJ, Li MT, Zhang CC, Zhang XR, Wu YT. Dietary administration of the Bacillus subtilis enhances immune responses and disease resistance in chickens. Frontiers in microbiology. 2020; 11:1768. https://doi.org/10.3389/fmicb.2020.01768 PMID: 32849392

71. Jay JM, Loessner MJ, Golden DA. Modern food microbiology (7th ed). New York, NY: Springer Science and Business Media. 2005.

72. Tannock GW. A special fondness for Lactobacilli. Applied and Environmental Microbiology. 2004; 70 (6):3189–3194. https://doi.org/10.1128/AEM.70.6.3189-3194.2004 PMID: 15184111

73. Langhout DJ, Schutte JB, V Leeuwen P, Wiebenga J, Tamminga S. Effect of dietary high- and low-methylated citrus pectin on the activity of the ileal microflora and morphology of the small intestinal wall of broiler chicks. British poultry science. 1999; 40(3):340–347. https://doi.org/10.1080/00071669987421 PMID: 10475630

74. Lee JT, Bailey CA, Cartwright AL. beta-Mannanase ameliorates viscosity-associated depression of growth in broiler chickens fed guar germ and hull fractions. Poultry science. 2003; 82(12):1925–1931. https://doi.org/10.1093/ps/82.12.1925 PMID: 14717550

75. Smulikowska S, Czerwiński J, Mieczkowska A. Effect of an organic acid blend and phytase added to a rapeseed cake-containing diet on performance, intestinal morphology, caecal microflora activity and thyroid status of broiler chickens. Journal of animal physiology and animal nutrition. 2010; 94(1):15–23. https://doi.org/10.1111/j.1439-0396.2008.00876.x PMID: 19138346

76. Ahir VB, Koringa PG, Bhatt VD, Ramani UV, Tripathi AK, Singh KM, et al. Metagenomic analysis of poultry gut microbes. Indian Journal of Poultry Science. 2010; 45(2):111–114.

77. Zhang S, Zhong G, Shao D, Wang Q, Shi S. Dietary supplementation with Bacillus subtilis promotes growth performance of broilers by altering the dominant microbial community. Poultry science. 2021; 100(3):100935. https://doi.org/10.1016/j.psj.2020.12.032 PMID: 33652528

78. Corrigan A, Horgan K, Clipson N, Murphy RA. Effect of dietary supplementation with a Saccharomyces cerevisiae mannan oligosaccharide on the bacterial community structure of broiler cecal contents. Applied and environmental microbiology. 2011; 77(18):6653–6662. https://doi.org/10.1128/AEM.05028-11 PMID: 21803917

79. Emami NK, Daneshmand A, Naeini SZ, Graystone E, Broom L. Effects of commercial organic acid blends on male broilers challenged with E. coli K88: Performance, microbiology, intestinal morphology, and immune response. Poultry science. 2017; 96(9):3254–3263. https://doi.org/10.3382/ps/pex106 PMID: 28453753

80. Lu L, Guo J, Li S, Li A, Zhang L, Liu Z, et al. Influence of Phytase Transgenic Corn on the Intestinal Microflora and the Fate of Transgenic DNA and Protein in Digesta and Tissues of Broilers. PloS one. 2015; 10(11):0143408. https://doi.org/10.1371/journal.pone.0143408 PMID: 26599444

81. Chen S, Cheng H, Wyckoff KN, He Q. Linkages of Firmicutes and Bacteroidetes populations to methanogenic process performance. Journal of industrial microbiology & biotechnology. 2016; 43(6):771–781. https://doi.org/10.1007/s10295-016-1760-8 PMID: 27021844

82. Zhang Y, Liu Y, Li J, Xing T, Jiang Y, Zhang L, et al. Dietary corn-resistant starch suppresses broiler abdominal fat deposition associated with the reduced cecal Firmicutes. Poultry science. 2020; 99 (11):5827–5837. https://doi.org/10.1016/j.psj.2020.07.042 PMID: 33142500

83. S Serajus, K Seon-Woo, Haley BJ, Van K, B Debabrata. Alternative Growth Promoters Modulate Broiler Gut Microbiome and Enhance Body Weight Gain. Frontiers in microbiology. 2017; 8:2088. https://doi.org/10.3389/fmicb.2017.02088 PMID: 29123512

84. Duncan SH, Hold GL, Harmsen H, Stewart CS, Flint HJ. Growth requirements and fermentation products of Fusobacterium prausnitzii, and a proposal to reclassify it as Faecalibacterium prausnitzii gen. International journal of systematic and evolutionary microbiology. 2002; 52(6):2141–2146. https://doi.org/10.1099/00207713-52-6-2141

85. Abaidullah M, Peng S, Kamran M, Song X, Yin Z. Current Findings on Gut Microbiota Mediated Immune Modulation against Viral Diseases in Chicken. Viruses. 2019; 11(8):681. https://doi.org/10.3390/v11080681 PMID: 31349568

86. Biddle A, Stewart L., Blanchard J., and Leschine S. Untangling the Genetic Basis of Fibrolytic Specialization by Lachnospiraceae and Ruminococcaceae in Diverse Gut Communities. Diversity. 2013; 5 (3):627–640. https://doi.org/10.3390/d5030627

87. Parker BJ, Wearsch PA, Veloo AV, Rodriguez-Palacios A. The Genus Alistipes: Gut Bacteria with Emerging Implications to Inflammation, Cancer, and Mental Health. Frontiers in immunology. 2020; 11:906. https://doi.org/10.3389/fimmu.2020.00906 PMID: 32582143

88. Pichler MJ, Yamada C, Shuoker B, Alvarez-Silva C, Gotoh A, Leth ML, et al. Butyrate producing colonic Clostridiales metabolise human milk oligosaccharides and cross feed on mucin via conserved pathways. Nature communications. 2020; 11(1):3285. https://doi.org/10.1038/s41467-020-17075-x PMID: 32620774

89. Zhang J, He X, Zhang H, Liao Y, Wang Q, Li L, et al. Factors Driving Microbial Community Dynamics and Potential Health Effects of Bacterial Pathogen on Landscape Lakes with Reclaimed Water Replenishment in Beijing, PR China. International journal of environmental research and public health. 2022; 19(9):5127. https://doi.org/10.3390/ijerph19095127 PMID: 35564521

90. Muñoz M, Guerrero-Araya E, Cortés-Tapia C, Plaza-Garrido A, Lawley TD, Paredes-Sabja D. Comprehensive genome analyses of Sellimonas intestinalis, a potential biomarker of homeostasis gut recovery. Microbial genomics. 2020; 6(12): 000476. https://doi.org/10.1099/mgen.0.000476 PMID: 33206037

91. Xu Y, Yu Y, Shen Y, Li Q, Lan J, Wu Y, et al. Effects of Bacillus subtilis and Bacillus licheniformis on growth performance, immunity, short chain fatty acid production, antioxidant capacity, and cecal microflora in broilers. Poultry science. 2021; 100(9):101358. https://doi.org/10.1016/j.psj.2021.101358 PMID: 34358955

92. Zhou Z, Nie K, Huang Q, Li K, Sun Y, Zhou R, et al. Changes of cecal microflora in chickens following Eimeria tenella challenge and regulating effect of coated sodium butyrate. Experimental parasitology. 2017; 177:73–81. https://doi.org/10.1016/j.exppara.2017.04.007 PMID: 28455119

93. Wu D, Wu SB, Choct M, Swick RA. Performance, intestinal microflora, and amino acid digestibility altered by exogenous enzymes in broilers fed wheat- or sorghum-based diets. Journal of animal science. 2017; 95(2):740–751. https://doi.org/10.2527/jas.2016.0411 PMID: 28380608

94. Kim M, Ingale SL, Hosseindoust A, Choi Y, Kim K, Chae B. Synergistic effect of exogenous multi-enzyme and phytase on growth performance, nutrients digestibility, blood metabolites, intestinal microflora and morphology in broilers fed corn-wheat-soybean meal diets. Animal bioscience. 2021; 34 (8):1365–1374. https://doi.org/10.5713/ab.20.0663 PMID: 33561925

95. Ballou AL, Ali RA, Mendoza MA, Ellis JC, Hassan HM, Croom WJ, et al. Development of the Chick Microbiome: How Early Exposure Influences Future Microbial Diversity. Frontiers in veterinary science. 2015; 3:2. https://doi.org/10.3389/fvets.2016.00002 PMID: 26835461

96. Tolonen AC, Petit E, Blanchard JL, Warnick TA, Leschine SB. Technologies to study plant biomass fermentation using the model bacterium Clostridium phytofermentans. In: Sun J, Ding SY, Doran-Peterson J (eds) Biological conversion of biomass for fuels and chemicals: explorations from natural utilization systems. The Royal Society of Chemistry Energy and Environment series. 2014; 10:114–139 https://doi.org/10.1039/9781849734738-00114

97. Van DAP, Moens F, Pignataro G, Schnurr J, Ribecco C, Gramenzi A, et al. Yeast-derived formulations are differentially fermented by the canine and feline microbiome as assessed in a novel in vitro colonic

fermentation model. Journal of agricultural and food chemistry. 2020; 68(46):13102–13110. https://doi.org/10.1021/acs.jafc.9b05085 PMID: 31909618

98. Cui Y, Zhang L, Wang X, Yi Y, Shan Y, Liu B, et al. Roles of intestinal Parabacteroides in human health and diseases. FEMS microbiology letters. 2022; 369(1):072. https://doi.org/10.1093/femsle/fnac072 PMID: 35945336