Effects of probiotic supplements on growth performance and intestinal microbiota of partridge shank broiler chicks

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Background The benefits of probiotics being used in animals are well-documented via evidenced growth performance improvement and positive modulations of gut microbiota (GM). Thus, a combination of effective microorganisms (EM) has been frequently used in animal production, including broilers. However, there are only very limited reports of EM on the growth performance and the modulation in GM of partridge shank broiler chicks.
Methods: We attempted to evaluate the effects of a basal diet with the addition of an EM mixture on the growth performance and gut microbiome of the chicks. A total of 100 ten-day-old female partridge shank broiler chicks were randomly divided into two groups of 50 chicks each, of which, one group fed with EM supplementation in the basal diet (designated as EM-treated group), the other group just fed with a basal diet (referred as to non-EM treated group or control group). The body weight, daily feed intake, daily gain, feed conversion ratio and other growth parameters were observed and compared between EM-treated and non-EM-treated chicks, and the gut microbiota was profiled by 16S rRNA -based next generation sequencing (NGS). **Results** Chicks fed with a basal diet with the addition of EM showed significantly increased performances in body weight (BW), average daily gain (ADG) and reduced feed conversion ratio (FCR). Histological observation indicated that dietary supplementation of EM significantly increased the villus heights (VH) and the ratio of villus height to crypt depth (VH/CD), while decreased the CD of jejunum, ilea, and ceca. The results of 16S rRNA -based gut microbiota analyses showed that *Firmicutes* accounted for the most of the relative abundance (63.24%~92.63%), followed by *Proteobacteria* (0.62%~23.94%), *Bacteroidetes* (0.80%~7.85%), *Actinobacteria* (0.06%~13.69%) and others in both EM-treated and non-EM-treated broiler chicks. The addition of EM could not alter the alpha diversity of gut microbiota. Compared with the non-EM-treated chicks, the abundances of bad bacteria in the phyla of *Firmicutes*,

Euryarchaeota, and *Ruminococcus* were dramatically decreased in that of EM-treated chicks, while the abundances of good bacteria in the phyla of *Actinobacteria* and *WPS-2* were significantly increased. **Conclusions:** The supplementation of EM in feed could improve the growth performance and positively influence the morphological characteristics of the intestine, and ameliorate the community and structure of the intestinal microbiota of partridge shank broiler chicks.

Article

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Short title: Effects of probiotics on partridge shank broilers

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ABSTRACT

Background: The benefits of probiotics being used in animals are well-documented via evidenced growth performance improvement and positive modulations of gut microbiota (GM). Thus, a combination of effective microorganisms (EM) has been frequently used in animal production, including broilers. However, there are only very limited reports of EM on the growth performance and the modulation in GM of partridge shank broiler chicks.

Methods: We attempted to evaluate the effects of a basal diet with the addition of an EM mixture on the growth performance and gut microbiome of the chicks. A total of 100 ten-day-old female partridge shank broiler chicks were randomly divided into two groups of 50 chicks each, of which, one group fed with EM supplementation in the basal diet (designated as EM-treated group), the other group just fed with a basal diet (referred as to non-EM treated group or control group). The body weight, daily feed intake, daily gain, feed conversion ratio and other growth parameters were observed and compared between EM-treated and non-EM-treated chicks, and the gut microbiota was profiled by 16S rRNA -based next generation sequencing (NGS).

Results: Chicks fed with a basal diet with the addition of EM showed significantly increased performances in body weight (BW), average daily gain (ADG) and reduced feed conversion ratio (FCR). Histological observation indicated that dietary supplementation of EM significantly increased the villus heights (VH) and the ratio of villus height to crypt depth (VH/CD), while decreased the CD of jejunum, ilea, and ceca. The results of 16S rRNA -based gut microbiota analyses showed that *Firmicutes* accounted for the most of the relative abundance (63.24%~92.63%), followed by *Proteobacteria* (0.62%~23.94%), *Bacteroidetes* (0.80%~7.85%),

Actinobacteria (0.06%~13.69%) and others in both EM-treated and non-EM-treated broiler chicks. The addition of EM could not alter the alpha diversity of gut microbiota. Compared with the non-EM-treated chicks, the abundances of bad bacteria in the phyla of *Firmicutes*, *Euryarchaeota*, and *Ruminococcus* were dramatically decreased in that of EM-treated chicks, while the abundances of good bacteria in the phyla of *Actinobacteria* and *WPS-2* were significantly increased.

Conclusions: The supplementation of EM in feed could improve the growth performance and positively influence the morphological characteristics of the intestine, and ameliorate the community and structure of the intestinal microbiota of partridge shank broiler chicks.

Subjects: Food Science and Technology, Zoology, Microbiology

Keywords: Probiotics; Effective Microorganisms; Growth Performance; Microbiota; Partridge Shank Broiler

INTRODUCTION

Feed cost accounts for about 70%~80% of the total cost of poultry production. Thus, great efforts have been ~~paid on the improvement of~~ nutritive values of feeds to enhance growth performance and health of animals (Ahmad et al., 2018). Probiotics, defined as “live microorganisms”, are one of the major feed additives routinely being used in animal production for decades due to the ~~confer~~ health benefits to the host when administered in an adequate amount (FAO, 2002; Markowiak et al., 2018, Iriti et al., 2019; Reszka et al., 2020). For poultry, probiotics could improve feed intake and digestion efficiency by increasing the activity of

digestive enzyme, keep the balance of bacteria in gastrointestinal (GI) tract, promote the gut integrity and thus improve the growth performance and health of chicks (Johnson et al., 2018; Soomro et al., 2019; Hack et al, 2020). Patidar (1999) showed the effect of Lactobacilli on increased the titers of haemagglutination inhibition antibody of chicks after feeding for 3-4 weeks (Patidar et al., 1999). Vinayasree (2012) evaluated the probiotic organisms on the performance of broilers, and found the use of probiotics faecal coliform count at the end of 6th week in experiment group was significantly lower when compared to the control groups (Vinayasree et al., 2012). Fazelnia (2021) showed the effects of dietary supplementation of potential probiotics *bacillus subtilis*, *bacillus licheniformis*, and *saccharomyces cerevisiae* and synbiotic improves the growth performance and immune responses on broiler chickens, and they found feeding synbiotic and probiotic alleviated the negative effects of S. typhimurium on growth and immunity of broiler chicks (Fazelnia et al., 2021). Relevant studies were reported on the effects of different probiotics supplementation in diet in broilers production (Zuanon et al., 1998; Ergun et al., 2000; Vicente et al., 2007).

In 1991, Terou Higa reported a multifunctional microbe flora composed of more than 80 kinds of microorganisms, named as Effective Microorganisms (EM) (Aruoma et al., 2002). The dominant bacteria in the EM are *Lactobacillus*, photosynthetic bacteria, *Actinomycetes*, yeasts and filamentous bacteria. Nowadays, EM has been widely used in more than 40 countries and/or areas, including Japan, the United States, India and China (Rybarczyk et al., 2016; Li et al., 1994). Previous researches demonstrated that EM can also improve soil performance, promoting crop growth and enhancing plant stress resistance. Investigations on broilers carried out by

Chantsawang showed that EM could increase body weight, feed intake, feed conversion efficiency, and immune response of EM-treated chicks (Chantsawang et al., 1999). Safalaoh (2006) conducted a study on the effect of EM on body weight gain, dressing percentage, abdominal fat and serum cholesterol content of broilers by supplementing EM in drinking water, and it was found that birds feed with EM had higher weight gains, feed efficiency, while lower feed intake and serum cholesterol content than that in control birds (Safalaoh et al., 2006). Besides, EM also has a beneficial effect on promoting animal growth and health. Further studies showed that EM could also improve meat quality, increase slaughter rate, and reduce the rate of death in economic animals (Jagdish et al., 1993; Alvarez et al., 1994; Silva et al., 2000; Patterson et al., 2003; Alagawany et al., 2018; Abd et al., 2020). However, controversy was existed in EM effect on broilers growth performance. Wondmeneh found the supplementation of EM in chicken's feed had no significant effect on mortality, feed conversion ratio (FCR) and weight gain (Wondmeneh et al., 2011).

Partridge shank chick, a local broiler breed in China, is a relatively smaller body size chick with the features of tender meat and high nutritional value as well as a special flavor when cooked. Therefore, it is very well favored by consumers in China. According to the previous reports, local breeds of broiler chick account for 46.52% of broiler slaughter in China in 2017, and this proportion was continuously increased in 2018 (Zhao et al., 2019). However, the growth rate of partridge shank broiler chick is slow. This characteristic may attribute to its genetic basis, the environmental factor(s), nutrition, and so on. It was found that the gut is an important site of nutrient absorption in animals, and better development of the intestinal system could benefit the

nutrient absorption and improve animal growth performance and health (Mekbungwan et al., 2004). However, we have limited information on the development of intestinal microbiota of partridge shank broiler chicks. And the knowledge on the effect of EM on broilers, especially in partridge shank broilers is poor. We hypothesized that the EM would improve the growth performance and the structure and composition of gut microbiota, perhaps via a mechanism of inhibiting the colonization of bad bacteria. The aim of the study was to evaluate the effects of EM on the growth performance, gut health and microbiota of partridge shank broilers.

MATERIALS AND METHODS

Ethics Statement

All procedures involving live animals were verified and approved by the Office of Animal Care and Use of Jiangxi Agricultural University (protocol number JXAU-LL-20190022). The chicks used in this study were housed at the Animal Research Unit of Jiangxi Agricultural University, located at the college of Animal Science and Technology in Nanchang, Jiangxi, China.

Chicks and experimental design

Ten-day-old female partridge shank broiler chicks (n=100) were purchased from a local commercial hatchery. All of the chicks had been individually wing-tagged, and immunized with the vaccines against Marek's disease, Newcastle disease, and Infectious bursal disease in 1, 4 and 10 days old, respectively. The experiment was carried out in Jan, 2020, all broiler chicks had ad libitum access to feed and water, and the feed was offered four times daily at 06.00 am, 11.00

126 am, 16.00 pm, and 20.00 pm, respectively. The chicks were then randomly divided into 2
127 experimental groups, and each group included 5 repetitions with 10 chicks per replication. All
128 chicks of each replication were housed in 0.96 x 0.96 m chick coops (at the Chicken
129 Experimental Unit, no. 109, Jiangxi Agricultural University, China) under the same
130 environmental conditions, including a constant temperature of 28 to 31℃ and 20 h light access
131 throughout the experiment.
132 The nutrient levels of the basal diet (maize-soybean-based meal diet) corresponded to the
133 NRC (1994) recommended requirements for broilers (Table 1). Chickens in experimental group,
134 designated as EM-treated group (abbreviated as group EM), were fed a basal diet supplemented
135 with 0.5 ml (about 2.5 x 10^9 colony-forming unit) EM per chick/day for 20 days and the chicks
136 in the negative control group, designated as non-EM-treated group (abbreviated as group B) were
137 just fed by the basal diet for 20 days. The bacterial composition of EM used in this study was
138 determined by 16S rRNA sequencing on Illumina HiSeq 4000 platform, and the bacterial
139 background information of EM used is supplied in Supplementary Table S1. The initial and final
140 weights, daily feed intake of the chicks in each group were recorded, and the feces of five chicks
141 in each group were sampled at the 1st, 10th, and 20th experimental day. Then the feed intake was
142 daily measured, body weight (BW) gain was measured at the end of experiment and then these
143 parameters were used to calculate average daily intake (ADFI), average daily gain (ADG), and
144 feed/gain ratio (F/G). At the 20th experimental day, 5 chicks of each group were narcotized by
145 pentobarbital sodium and dissected, and the cecal contents were collected. For gut microbiota
146 profiling, excreta at the 1st, 10th and 20th experimental day and cecal contents at the 20th

147 experimental day were collected in both groups (designated as EM0, EM10, EM20, and EM20C
148 for samples from EM-treated chicks; and B0, B10, B20, and B20C for samples from non-EM-
149 treated chicks).

150 **Histological observation**

151 At necropsy, different sections of intestines were examined and collected for histological
152 observation according the previous methods in our lab (Zhang *et al*, 2020). For each tissue
153 section, at least ten villi and crypts were measured using the cellSens Standard system (Olympus,
154 Japan) with villous height (VH) and crypt depth (CD), which would be used for the calculation
155 of VH/CD ratio.

156 **Bacterial DNA extraction and 16S rRNA gene sequencing**

157 Bacterial genomic DNA were extracted by the QIAamp DNA Stool Mini Kit (Qiagen, Hilden,
158 Germany) and quantified according to the previous method (Song et al., 2017). Amplification of
159 the hypervariable V4 region of 16S rRNA gene was performed by using 'universal' primers 515F
160 (5'-GTGYCAGCMGCCGCGTAA-3') and 806R (5'-GGACTACNVGGGTWTCTAA-3') flanked
161 with adapter and barcode sequences (Kuczynski et al., 2011). The PCR was carried out under the
162 following conditions: 95℃ for 5 min; 25 cycles of: 95℃ for 30 s, 56℃ for 45 s, 72℃ for 30 s; a
163 final extension for 10 min, and then hold at 4℃. The amplicons were cleaned by using AMPure
164 XP beads (Beckman Coulter, Brea, CA, USA), and then normalized, pooled with the adapters
165 and the dual indices using the Nextera XT Index Kit (Cat No.: FC-131-2001, Illumina, San
166 Diego, CA, USA). A second PCR amplification with 5 cycles were executed with Nextera XT

Index primers in following conditions: 95℃ for 4 min; 5 cycles of: 95℃ for 30 s, 55℃ for 40 s, 72℃ for 40 s; a final extension for 5 min, and then hold at 4℃. The PCR products were cleaned up again with AMPure XP beads, and thus the sequencing libraries were established. The libraries were validated to the expected size of about 440 bp on a Bioanalyzer trace for the final library. The libraries were quantified using a Qubit 4.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) according to the fluorometric quantification method using dsDNA binding dyes. The concentration of each DNA library was determined by an Agilent Technologies 2100 Bioanalyzer. For sequencing, the individual library was diluted for 4 nM, and aliquoted with 5 μl of diluted DNA was then mixed for pooling libraries and sequenced on the Illumina Hiseq 4000 platform in paired-end (PE) technology at 2 x 250 nt using Illumina v2 kit (Illumina, San Diego, CA, USA) in Guhe Information Co., Ltd in Hanzhou, China.

Metagenomic analysis

The raw reads from 16S rRNA sequencing were automatically input for quality control, trimming, demultiplexing of samples and generating fastq files. Afterwards, the reads were subjected to further proceeding by pipeline QIIME 2 (http://qiime.org/). Operational taxonomic units (OTUs), included de-replication, cluster, detection of chimera, were picked using Vsearch v1.11.1 based on a 97% 16S rRNA gene sequence identity level (Rognes et al., 2016). Taxonomic assignment of individual datasets was determined at several taxonomic levels: kingdom, phylum, class, order, family, genus, and species by using SILVA 128 (Quast et al., 2013). OTUs classified as chloroplasts or mitochondria were subsequently removed. The

obtained sequences classified as bacteria and archaea were examined with BLAST (Basic Local Alignment Search Tool) (Mount, 2007).

Alpha diversity was calculated with QIIME, including index of observed species, chao1, shannon, simpson, and PD_whole_tree. Beta diversity was performed using QIIME with the matrix of weighted and unweighted Unifrac distance. LEfSe analysis was performed by using linear discriminant analysis (LDA) to estimate the different size of the effect of abundance of each component (species), and to identify communities or species that had significant differences in the classification of the samples (Segata et al., 2011).

Statistical analysis

The differences of data between EM-treated group and non-EM-treated group were analyzed by student t test in SPSS 26.0 (IBM, USA). The replicate was defined as the experimental unit. Comparisons of parameters of growth performance across the groups were carried out by one-way analysis of variance (ANOVA) and significant differences among group means were determined using the least significant difference (LSD) test. The beta diversity indices were calculated based on the principal co-ordinates analysis (PCoA) method (Quinn Gp, 2002). Kruskal-Walls test was used to identify the difference of alpha diversity indices and bacterial species which showed significant differences between different groups by R stats package. A p-value of <0.05 was set as the statistically significant level.

RESULTS

Effects of EM on growth performance of partridge shank broiler chicks

In this study, addition of EM significantly increased the BW, ADG and decreased the FCR at the 10th ($P < 0.001$) and 20th ($P < 0.05$) experimental-day when compared with the controls (Table 2). The BW gain for all the evaluated periods (day 0 to 10, day 0 to 20) was improved for chicks supplemented with EM. Similarly, ADG from day 0 to 10, from day 11 to 20 and overall period (day 0 to 20) were increased in EM-treated chicks. Moreover, FCR was decreased during day 0 to 10, and day 11 to 20, while no significance difference in all evaluated period. While the EM addition did not have significant impact on ADFI.

Effects of EM on the morphology of intestines of partridge shank broiler chicks

Dietary supplementation of EM significantly increased the jejunal villus height ($P < 0.001$), ratio of jejunal villus height to crypt depth (VH/CD, $P < 0.001$) but decreased the jejunal crypt depth ($P < 0.001$). Furthermore, EM supplementation remarkably increased both ileal ($P < 0.001$) and cecal ($P < 0.001$) villus height and ratio of VH/CD ($P < 0.001$), but decreased ileal ($P < 0.05$) and cecal ($P < 0.05$) crypt depth (Table 3).

Microbial diversity of excreta and cecal microbiota of partridge shank broiler chicks

The Illumina HiSeq 4000 sequencing was performed using 40 samples and a total of 3,505,030 raw sequence reads were generated. After quality control, 3,234,992 (92.30%) clean reads were obtained, with an average of 80,874 clean sequences per sample (supplementary Table S2). Shannon, Simpson and Chao1 indices were employed to evaluate the alpha diversity within the sequence datasets based on the observed OTUs. Of the alpha diversity indices, no significant

variation was observed between the comparable groups B0 and EM0, B10 and EM10, B20 and EM20, B20C and EM20C, indicating EM had limited influence on the alpha diversity indices (Table 4). The beta diversity among groups was presented on principal co-ordinates analysis (PCoA) to distinguish the microbial communities (Figure 1). The results revealed that the microbial communities in cecal contents showed a striking distinctness with that in excreta. Clusters of excreta microbiota were superimposed over the PCoA analysis and represented the differences among the groups.

Comparison of microbial communities of excreta microbiota between EM-treated and non-EM-treated chicks

In the composition analysis at the phylum level, *Firmicutes* accounted for the most of the relative abundance (63.24%~92.63%), followed by *Proteobacteria* (0.62%~23.94%), *Bacteroidetes* (0.80%~7.85%), *Actinobacteria* (0.06%~13.69%) and others. With increasing age, the abundance of *Firmicutes* tended to decrease and the abundance of *Proteobacteria*, *Bacteroidetes* and *Actinomycete* tended to increase (Figure 2 and Table S3). At the genus level, *Lactobacillus* had the highest relative abundance in the excreta samples (33.26%~78.03%), followed by *Streptococcus* (0.01%~19.07%), *Enterococcus* (0.16%~20.94%), and *Bacteroides* (0.46%~5.27%). Similarly, the abundances of *Lactobacillus* in feces in both EM-treated and non-EM-treated broiler chicks were reduced, while the abundances in EM-treated chicks were higher than that in non-EM-treatedled *chick*s. However, in cecum samples, an unclassified genus from the family *Lachnospiraceae* accounted for the most of the relative abundance (24.02%~36.41%),

247 followed by unclassified genus from the order *Clostridiales* (20.32%~23.40%), unclassified
248 genus in the family *Lachnospiraceae* (6.50%~7.30%), and *Ruminococcus* (2.82%~4.63%)
249 (Figure 3 and Table S4).

250 **Comparison of gut microbiota landscape in non-EM-treated chicks**

251 To explore the gut microbiota landscape of the boiler chicks in non-EM-treated group, ANOVA
252 test was performed. At the phylum level, the abundances of *Firmicutes* were significantly
253 decreased (90.53% to 63.24%) from B0 (10d age) to B20 (30d age). While the abundances of
254 *Euryarchaeota*, *Synergistetes*, *Verrucomicrobia*, and *Actinobacteria* were significantly increased
255 as the chicks grew up (Table 5 and Figure S1A). At the genus level, abundances of *Prevotella*,
256 *Coprococcus*, *Desulfovibrio, Gallibacterium*, and *Acinetobacter* tended to be increased from age
257 10d to 30d (Figure S1B).

258 **Comparison of gut microbiota landscape in EM-treated chicks**

259 Among the EM-treated partridge shank broiler chicks, four kinds of gut bacteria at the phylum
260 level were significantly different among growth stages of EM0, EM10 and EM20. Bacteria in
261 *Proteobacteria*, *Synergistetes, and WPS-2* were significantly increased from EM0 to EM20
262 (Table 6 and Figure S2A). At the genus level, the abundances of *Methanobrevibacter*,
263 *Enterococcus*, *Streptococcus,* and *Gallibacterium* were significantly augmented in EM10, while
264 decreased in EM20. *Faecalibacterium* and *Megamonas* were reduced as the time of EM-treated
265 (Figure S2B).

Comparison of excretal and cecal microbiota between EM-treated and non-EM-treated chicks

To address the impacts of EM on the structure and abundance of microbiota in feces and cecal contents, the abundances of fecal and cecal bacteria in chicks at the end of the experiment were analyzed. For the excretal microbiota, the abundances of two bacteria *TM7* ($P < 0.01$)and *Tenericutes* ($P < 0.01$)at the phylum level and one at the genus level *Acinetobacte* ($P < 0.05$) were reduced in group EM20 when compared with that in group B20 (Table 7 and Figure S3). As the normal structure of bacterial communities in ceca was very different from that in excreta, the changes of cecal microbiota in EM-treated broiler chicks were different. When compared with the control group, the abundances of *Firmicutes* ($P < 0.001$), *Euryarchaeota* ($P < 0.05$), and *Ruminococcus* ($P < 0.05$) were significantly reduced, while the abundances of *Actinobacteria* ($P < 0.001$) and *WPS-2* ($P < 0.001$) were significantly increased (Table 8 and Figure S4).

DISCUSSION

As reported from previous studies, supplements with probiotics in feed could improve the feed intake, weight gain and feed efficiency in broilers (Waititu et al., 2014; Qorbanpour et al., 2018; Jha et al., 2020). Therefore, in order to enhance the growth rate, maintain intestinal integrity, and improve the overall health status of chicks in intensive production conditions, the use of probiotic preparations as a supplement is a common practice in animal production (Wondmeneh et al., 2011). In this study, an EM mixture containing multiple species of bacteria, of which most

are naturally existing beneficial microorganisms, including both oxybiotic and anaerobic microbes, was applied to evaluate the effects on the growth performance and gut health of partridge shank broiler chicks. Researchers have reported that probiotics had positive effects on BW and ADG of animals (Huang et al., 2019; Tao et al., 2021). The functional inconsistency of probiotics among these studies, including the present study, might attribute to the type, dosage of probiotics being used, and the breeds of the broilers as well.

In this study, positive effects of the EM supplementation on BW, ADG and FCR were found in partridge shank broiler chicks. The BW gain and ADG were significantly higher in EM-fed chicks than that in control chicks both at the first pahse (0-10th day) and second phase (11-20th day). ~~While the~~ ADFI showed no difference between the EM-fed and control chicks ~~at~~ both ~~two~~ phases~~.~~ ~~W~~hich ~~indicated~~ the EM supplementation could improve the feed conversion efficiency and led to the decrease of FCR. These findings agree with previous studies regarding the beneficial effects of EM and probiotics on the growth performance and gut health of partridge shank broiler chicks (Chantsawang et al., 1999; Safalaoh., 2006; Alkhalf et al., 2010; Xu et al., 2014; Fazelnia et al., 2021). ~~Chantsawang~~ (1999) evaluated the effects of EM supplementation on 4 different types of poultry, and ~~was~~ found that EM additive could significantly increase~~d~~ breast percentage in Muscovy duck, and decrease~~d~~ ash content of breast meat in Arbor Acers broiler chicken~~s~~ ~~(Chantsawang et al., 1999)~~. Safalaoh (2006) showed that the addition of EM in diet ~~had~~ significantly ~~BW gains~~ (2094±11 g) and ADG ~~than those~~ on the control diet (2057±15 g) ~~in broilers~~ during 1-42 days of age ~~(Safalaoh., 2006)~~. On the other hand, there are reports which state that probiotics or EM ~~fed birds~~ had no role on the growth performance and mortality

in broilers. Mokhtari (2010) and Chen (2015) found reduced feed intake by the addition of probiotics and/or prebiotics in broiler diet (Mokhtari et al., 2010; Chen et al., 2015). Sarangi (2016) demonstrated that the use of probiotics in broiler diet did not affect FCR (Sarangi et al., 2016). In addition, Yousefi (2007) found the weight gain was not affected by supplementation of probiotics (Yousefi et al., 2007). The possible reasons might be the difference probiotic bacteria they used, and also might be related to several other factors such as bird breed, age, sex, and the dose rate of probiotics used (Kabir ., 2009; Sohail et al., 2012).

In this study, the EM additive positively influenced the morphological characteristics of the broiler's intestine. Histological observation indicated the supplementation of EM increased the height of intestinal villi in jejunum, ileum and cecum in the EM-fed broilers. The structure of intestinal villi are covered with the intestinal epithelium, under which there is a continuous cell layer of myofibroblasts that could regulate the epithelial renewal and defence processes (Ackermann et al., 1974). Furthermore, EM also increased the intestinal crypt depth (CD) and VH/CD rate of broilers. Crypts are associated with the proliferation of epithelial cells by producing defensins and dendocrine substances (Manning et al., 2004). Baum (2002) demonstrated that probiotics *Saccharomyces boulardii* and *Bacillus cereus* had beneficial effect on the epithelial structure and cryptic morphology (Baum et al., 2002). Awad (2009) evaluated the effect of addition of probiotics contented with *Lactobacillus salivarius* and *Lactobacillus reuteri* in feed significantly increased the BW, average daily weight gain, and improved the villus in small intestines, increased the VH/CD ratio in duodenum in broilers (Awad et al., 2009). The positive effects of EM used in this study contents multiple probiotic bacteria, such as

328 *Lactobacillus* (abundance of 84.02% ±12.31%, Table S1) and *Bacillus* (0.09% ± 0.11%),
329 which might benefit ~~for~~ the villus and cryptic morphology and ~~then~~ promote the intestinal health.
330 Probiotics are suitable for domestic animals, because they can inhibit the growth of pathogenic
331 bacteria and promote the growth of beneficial bacteria by producing different metabolites and
332 thus improve the gut microecological environment (Cummings and Kong, 2004; Attia et al.,
333 2013; Sun et al., 2019). Similar results were observed in the present study. Although the
334 abundances of *Lactobacillus* were reduced ~~with~~ the chicks ~~growing~~, the abundance of
335 *Lactobacillus* in EM-treated partridge shank broiler chicks were elevated when compared with
336 ~~that in~~ non-EM-treated broiler chicks. The abundance of *Acinetobacter* was significantly lower
337 in EM20 compared to that of B20. ~~As known, the most~~ members of *Acinetobacter* ~~were~~
338 enteropathogenic ~~agents of~~ infections (Michalopoulos and Falagas, 2010). Besides, the
339 commonly encountered pathogenic or zoonotic bacteria in poultry, such as E. coli, Streptococcus,
340 and ~~c~~lostridium were slightly reduced in the gut of EM-treated partridge shank broiler chicks. In
341 general, supplementation of EM in feed could ameliorate the community and structure of the
342 intestinal microbiota of partridge shank broiler chicks.
343

344 ## CONCLUSIONS

345 In this study, we observed that the feed supplemented with EM could increase the body weight
346 and average daily gain, and reduced feed conversion ratio, enhance intestinal integrity, and
347 balance the gut microflora of partridge shank broiler chicks. The findings could provide new
348 insights to improve the growth performance and the gut health of partridge shank broiler chicks.

ADDITIONAL INFORMATION AND DECLARATIONS

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Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

The individual contributions in the present study were as follows: conceptualization and methodology D. S., and Y. T.; validation Z. Y., Z. L., and P. W.; Investigation: Z. Y., B. Z., M. Z., and D. H.; data duration Y. Y., Z. D., G. W., and Q. W.; writing—original draft preparation Z. Y., and Z. L.; project administration D. S., and Q. W.; funding acquisition D. S. All authors have read and agreed to the published version of the manuscript.

Data availability statement

All sample raw reads were deposited at the Short Reads Archive (SRA) database belongs to the National Center for Biotechnology Information (NCBI) and are available under Bioproject ID PRJNA629019.

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Tables

Table 1 Ingredient composition of the basal diet being fed for the broiler chickens used in this study

Item	Amount (g/kg)
Ingredients	
Corn meal	581.5
Soybean meal	335
Soybean oil	32.1
Limestone	13
Dicalcium phosphate	20.5
L-lysine	3.4
DL-Methionine	1.5
Sodium chloride	3
Premix	10
Calculated nutrient levels	
Metabolizable energy (MJ/kg DM)	12.08
Crude protein (g/kg DM)	19.25
Calcium (g/kg DM)	1.07
Available phosphorus (g/kg DM)	4.6
Lysine (g/kg DM)	12.6
Methionine (g/kg DM)	4.27
Methionine + cysteine (g/kg DM)	8.35

DM: dry matter; Premix provided per kilogram of diet: vitamin A (all-trans-retinyl acetate), 10,000 IU; vitamin D3 (cholecalciferol), 3,000 IU; vitamin E (all-rac-α-tocopherol), 30 IU; menadione, 1.3 mg; thiamin, 2.2 mg; riboflavin, 8 mg; nicotinamide, 40 mg; choline chloride, 400 mg; calcium pantothenate, 10 mg; pyridoxine·HCl, 4 mg; biotin, 0.04 mg; folic acid, 1 mg; vitamin B12 (cobalamin), 0.013 mg; Fe (from ferrous sulphate), 80 mg; Cu (from copper sulphate), 8.0 mg; Mn (from manganese sulphate), 110 mg; Zn (from zinc oxide), 60 mg; I (from calcium iodate), 1.1 mg; Se (from sodium selenite), 0.3 mg.

568

569 **Table 2 Growth performance of Partridge Shank broiler chickens fed diets supplemented**

570 **with or without EM**

Item	EM-treated boilers	Non-EM treated boilers	p–value (ANOVA)
BW, g			
0 day	145.70±6.68	146.10±5.28	0.846
10th day	274.30±10.80	254.60±12.52	0.001**
20th day	563.40±32.22	533.10±19.55	0.020*
ADFI, g/day			
0-10 days	36.49 ± 8.54	35.47 ± 6.51	0.766
11-20 days	56.74 ± 7.58	53.39 ± 7.07	0.321
0-20 days	46.62 ± 13.02	44.43 ± 11.32	0.574
ADG, g/day			
0-10 days	12.86 ± 1.27	10.85 ± 1.07	0.001**
11-20 days	28.91 ± 3.03	27.85 ± 1.85	0.048*
0-20 days	20.89 ± 3.38	19.33 ± 2.04	0.025*
FCR			
0-10 days	2.84	3.27	0.021*
11-20 days	1.96	1.92	0.049*
0-20 days	2.40	2.59	0.053

571 BW=body weight; AVG=average; SD=Standard deviation; ADFI = average daily feed intake; ADG = average

572 daily gain; FCR= feed conversion ratio. * indicates 0.01 < p value < 0.05, ** indicates 0.01 < p value < 0.001.

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581 **Table 3 The villus height and crypt depth of intestine in chickens between EM-treated and**

582 **non-EM-treated negative control groups**

Item	Intestinal section	Average length ± standard deviation, μm		p-value (ANOVA)
		EM-treated	Control	
Villus height (VH)	Jejunum	575.35 ± 59.28	427.28 ± 52.80	0.000***
	Ileum	520.13± 42.93	342.79 ± 22.47	0.000***
	Cecum	82.83 ± 17.32	50.44 ± 9.27	0.000***
Crypt depth (CD)	Jejunum	39.55 ± 10.46	52.42 ± 11.88	0.001**
	Ileum	35.06 ± 10.14	42.03 ± 9.88	0.034*
	Cecum	19.01 ± 2.91	22.42 ± 4.86	0.011*
VH/CD value	Jejunum	15.45 ± 4.12	8.51 ± 2.06	0.000***
	Ileum	16.16 ± 5.69	8.64 ± 2.30	0.000***
	Cecum	4.46 ± 1.14	2.39 ± 0.79	0.000***

583 Note: * indicates 0.01 < p value < 0.05, ** indicates 0.001 < p value < 0.01, *** indicates p value < 0.001.

584

585 **Table 4 Microbiota alpha diversity among groups of chickens by Kruskal-Walls test**

Group	Shannon	Simpson	Chao1	Ace	Goods_coverage
B0	3.41±1.33	0.696±0.26	392.94±110.63	383.08±110.63	1.00±0.00
EM0	3.57±0.96	0.76±0.12	433.23±188.59	433.07±186.16	1.00±0.00
p-value	0.83	1.00	0.69	0.62	1.00
B10	3.51±1.09	0.74±0.10	538.89±209.50	536.24±203.14	1.00±0.00
EM10	3.17±1.44	0.69±0.25	584.54±201.78	563.80±198.39	1.00±0.00
p-value	0.69	1.00	0.69	0.83	1.00
B20	4.86±1.89	0.84±0.17	899.23±201.02	897.80±190.29	1.00±0.00
EM20	4.36±1.51	0.86±0.08	857.33±247.88	853.84±250.80	1.00±0.00
p-value	0.55	1.00	0.84	0.76	1.00
B20C	6.56±0.42	0.96±0.02	697.89±398.22	689.15±388.87	1.00±0.00
EM20C	6.41±0.63	0.96±0.03	1061.42±163.78	1054.42±159.89	1.00±0.00
p-value	1.00	0.84	0.15	0.09	1.00

586

587 **Table 5 Abundance differences of bacteria among the bird gut in non-EM-treated negative**

588 **control group**

Bacterial name	Average abundance, %			ANOVA test p value	Significance
	B0	B10	B20		
Firmicutes	90.5325	90.5611	63.2401	0.032	***
Euryarchaeota	0.0003	0.5090	0.1959	0.000	***
Synergistetes	0.0000	0.1370	1.2271	0.000	***
Verrucomicrobia	0.0001	0.0222	0.7400	0.000	***
Actinobacteria	0.0000	0.0003	0.0008	0.005	***
Methanobrevibacter	0.0003	0.4394	0.1830	0.000	***
Prevotella	0.0045	0.0701	0.2755	0.000	***
Streptococcus	0.0116	19.0748	6.4261	0.000	***
Coprococcus	0.0383	0.0906	0.4035	0.000	***
Desulfovibrio	0.0056	0.0047	0.0640	0.000	***
Gallibacterium	0.0000	0.0128	0.3822	0.000	***
Acinetobacter	0.0252	0.0022	1.1387	0.000	***

589

590 **Table 6 Abundance differences of bacteria among the gut of EM-treated broiler chickens**

Bacterial name	Average abundance, %			Variation test p value	Significance
	EM0	EM10	EM20		
Euryarchaeota	0.0001	0.4001	0.3005	0.0000	***
Proteobacteria	1.4001	8.5000	12.4002	0.0040	***
Synergistetes	0.0000	0.0002	0.9000	0.0000	***
WPS-2	0.0000	0.1000	0.4002	0.0000	***
Methanobrevibacter	0.0000	0.4000	0.3000	0.0000	***
Enterococcus	0.3001	20.9002	2.6001	0.0000	***
Streptococcus	0.0001	10.5001	5.2003	0.0000	***
Faecalibacterium	1.3000	0.2000	0.3000	0.6070	NA

Megamonas	1.2001	0.0001	0.1001	0.0000	***
Desulfovibrio	0.0001	0.3000	1.0000	0.0000	***
Gallibacterium	0.0000	3.7000	0.1000	0.0000	***

591 **Table 7 Abundance differences of bacteria in feces between EM-treated and non-EM-**
592 **treated negative control Partridge Shank broiler chickens**

Bacterial name	Average abundance, %		Variation test p value	Significance
	B20	EM20		
TM7	0.2001	0.0000	0.0021	**
Tenericutes	0.4001	0.1002	0.0021	**
Acinetobacter	1.1000	0.2000	0.0350	*

593

594 **Table 8 Abundance differences of bacteria in cecal contents between EM-treated and non-**
595 **EM-treated negative control Partridge Shank broiler chickens**

Bacterial name	Average abundance, %		Variation test p value	Significance
	B20C	EM20C		
Euryarchaeota	1.0003	0.3001	0.0431	*
Actinobacteria	0.8001	13.7001	0.0000	***
Firmicutes	91.1000	75.3000	0.0051	**
WPS-2	0.0002	0.7001	0.0000	***
Ruminococcus	15.8001	4.1001	0.0350	*

596

597 ## Figure Legends

598 **Figure 1. Principal component analysis (PCoA) based on the sequences from all samples**
599 **tested (A), excreta samples from the 0 (B), 10th (C), and 20th (D) experimental day, and**
600 **cecal content samples from the 20th experimental day (E).**

601 **Figure 2. Gut microbial composition at phylum-level.**

602 **Figure 3. Gut microbial composition at genus-level.**

603

604 Supporting Tables

605 **Table S1 Construction of EM used in this study.**

606 **Table S2 Summary of sequencing data obtained in this study.**

607 **Table S3 Abundances of intestinal flora between EM-treated and control group at the**

608 **phylum level.**

609 **Table S4 Abundance table of intestinal flora between EM-treated and control groups at the**

610 **genus level.**

611

612 Supporting Figures

613 **Figure S1 Excretal Bacteria with significant abundances among the bird gut in non-EM-**

614 **treated negative control group at phylum (A) and genus (B) level.**

615 **Figure S2 Excretal Bacteria with significant abundances among the bird gut in EM-treated**

616 **negative control group at phylum (A) and genus (B) level.**

617 **Figure S3 Excretal Bacteria with significant abundances between the EM-treated and non-**

618 **EM treated broilers at phylum (A) and genus (B) level.**

619 **Figure S4 Cecal Bacteria with significant abundances between the EM-treated and non-**

620 **EM treated broilers at phylum (A) and genus (B) level.**

Figure 1

Figure 1. Principal component analysis (PCoA) based on the sequences from all samples tested (A), excreta samples from the 0 (B), 10th (C), and 20th (D) experimental day, and cecal content samples from the 20th experimental day (E).

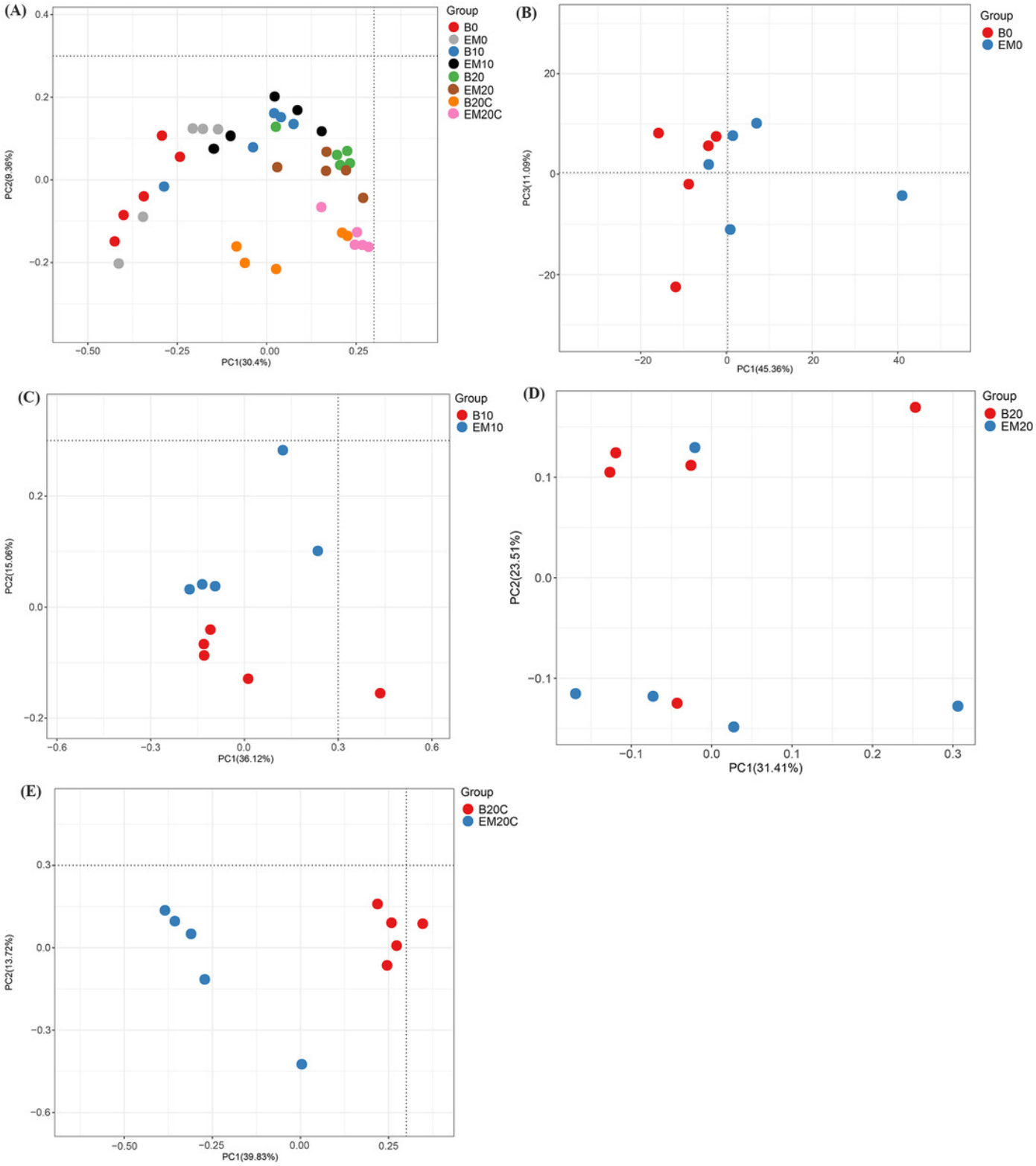
(A)
Group
B0
EM0
B10
EM10
B20
EM20
B20C
EM20C
PC2(9.36%)
PC1(30.4%)
(B)
Group
B0
EM0
PC3(11.09%)
PC1(45.36%)
(C)
Group
B10
EM10
PC2(15.06%)
PC1(36.12%)
(D)
Group
B20
EM20
PC2(23.51%)
PC1(31.41%)
(E)
Group
B20C
EM20C
PC2(13.72%)
PC1(39.83%)

Figure 2

Figure 2. Gut microbial composition at phylum-level.

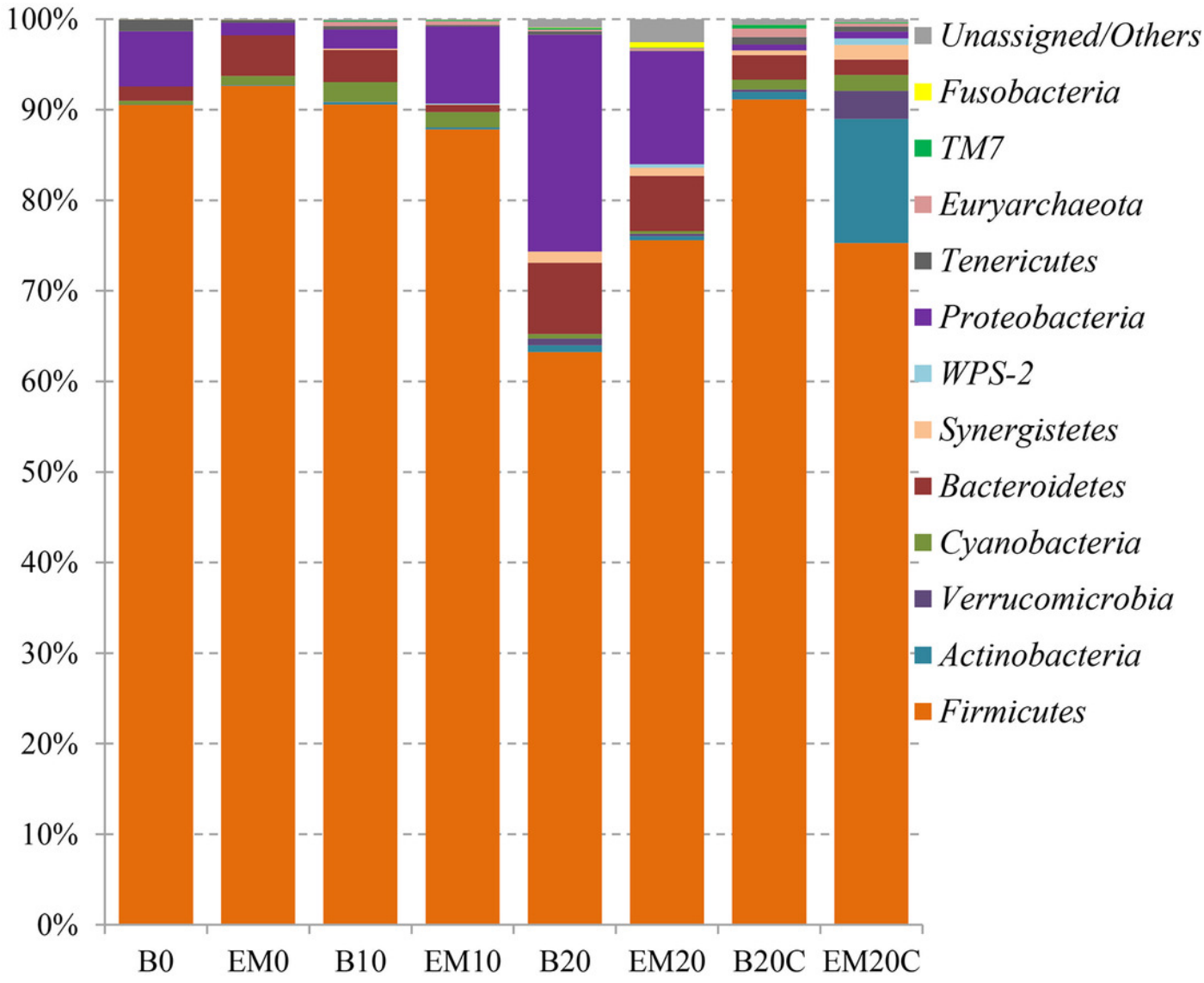

Figure 3

Figure 3. Gut microbial composition at genus-level.