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.

1 Article

2 **Effects of probiotic supplements on growth performance and gut**

3 **microbiota of partridge shank broiler chicks**

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22   **ABSTRACT**

23   **Background:** The benefits of probiotics being used in animals are well-documented via

24   evidenced growth performance improvement and positive modulations of gut microbiota (GM).

25   Thus, a combination of effective microorganisms (EM) has been frequently used in animal

26   production, including broilers. However, there are only very limited reports of EM on the growth

27   performance and the modulation in GM of partridge shank broiler chicks. This study aimed to

28   assess the effects of a basal diet with the addition of an EM mixture (84% of Lactobacillus) on

29   the growth performance, intestinal histomorphology and microbiome of the chicks.

30   **Methods:** A total of 100 ten-day-old female partridge shank broiler chicks were randomly

31   divided into two groups of 50 chicks each, of which, one group was fed with EM

32   supplementation in the basal diet (designated as EM-treated group), the other group was just fed

33   with a basal diet (referred as to non-EM treated group or control group). The body weight, daily

34   feed intake, daily gain, feed conversion ratio and other growth parameters along with intestinal

35   histomorphology were observed and compared between EM-treated and non-EM-treated chicks,

36   and the gut microbiota was profiled by 16S rRNA -based next generation sequencing (NGS)

37   during experimental periods.

38   **Results:** Chicks fed with a basal diet with the addition of EM showed significantly increased

39   performances in body weight (BW), and average daily gain (ADG), but reduced feed conversion

40   ratio (FCR). Histological observation indicated that dietary supplementation of EM significantly

41   increased the villus heights (VH) and decreased the crypt depth (CD) of jejunum, ilea, and ceca,

42   and thus increased the ratio of villus height to crypt depth (VH/CD). The results of 16S rRNA -

43   based gut microbiota analyses showed that *Firmicutes* accounted for the most of the relative

44   abundance (63.24%~92.63%), followed by *Proteobacteria* (0.62%~23.94%), *Bacteroidetes*

45   (0.80%~7.85%), *Actinobacteria* (0.06%~13.69%) and the others in both EM-treated and non-

46   EM-treated broiler chicks. The addition of EM could not alter the alpha diversity of gut

47   microbiota. Compared with the non-EM-treated chicks, the abundances of bad bacteria in the

48   phyla of *Firmicutes*, *Euryarchaeota*, and *Ruminococcus* were dramatically decreased in that of

49   EM-treated chicks, while the abundances of good bacteria in the phyla of *Actinobacteria* and

50   *WPS-2* were significantly increased.

51   **Conclusions:** The supplementation of the EM in feed could improve the growth performance

52   and positively influence the histomorphological characteristics of the intestine, and ameliorate

53   the community and structure of the intestinal microbiota of partridge shank broiler chicks.

54   **Subjects:** Food Science and Technology, Zoology, Microbiology

55   **Keywords**: Probiotics; Effective Microorganisms; Growth Performance; Microbiota; Partridge

56   Shank Broiler

57

58   **INTRODUCTION**

59   Feed cost accounts for about 70%~80% of the total cost of poultry production. Thus, great

60   efforts have been made to improve the nutritive values of feeds to enhance growth performance

61   and health of animals (Ahmad et al., 2018). Probiotics, defined as "live microorganisms", are

62   one of the major feed additives routinely being used in animal production for decades due to the

63   conferred health benefits to the host when administered in an adequate amount (FAO, 2002;

64 Markowiak et al., 2018, Iriti et al., 2019; Reszka et al., 2020). For poultry, probiotics could

65 improve feed intake and digestion efficiency by increasing the activity of digestive enzymes,

66 keep the balance of bacteria in gastrointestinal (GI) tract, promote the gut integrity and thus

67 improve the growth performance and health of birds (Johnson et al., 2018; Soomro et al., 2019;

68 Hack et al, 2020). Patidar et al. (1999) showed that *Lactobacilli* on increased the titers of

69 hemagglutination inhibition antibodies of chicks after feeding for 3-4 weeks. Vinayasree et al.

70 (2012) evaluated the effect of probiotic organisms on the performance of broilers, and found

71 with the use of probiotics fecal coliform bacteria counted at the end of 6th week in experiment

72 group were significantly lower when compared to the control groups. Fazelnia et al. (2021)

73 showed that the dietary supplementation of potential probiotics *Bacillus subtilis*, *Bacillus*

74 *licheniformis*, and *Saccharomyces cerevisiae* and synbiotic improved the growth performance

75 and immune responses of broiler chicks. Additionally, supplementation of synbiotic and

76 probiotic alleviated the negative effects of *S. typhimurium* on growth and immunity of broiler

77 chicks. Also other studies have reported beneficial effects of different probiotics on the broiler

78 growth (Zuanon et al., 1998; Ergun et al., 2000; Vicente et al., 2007).

79   In 1991, Terou Higa reported a multifunctional microbe flora composed of more than 80 kinds

80 of microorganisms, named as Effective Microorganisms (EM) (Higa., 1991; Aruoma et al.,

81 2002). The dominant bacteria in the EM are *Lactobacillus*, photosynthetic bacteria,

82 *Actinomycetes*, yeasts and filamentous bacteria. Nowadays, EM has been widely used in more

83 than 40 countries and/or areas, including Japan, the United States, India and China (Rybarczyk et

84 al., 2016; Li et al., 1994). Previous studies demonstrated that EM can also improve soil

85   performance, promoting crop growth and enhancing plant stress resistance. Investigations on

86   broilers carried out by Chantsawang et al. (1999) showed that EM could increase body weight,

87   feed intake, feed conversion efficiency, and immune response of EM-treated chicks. Safalaoh et

88   al. (2006) conducted a study on the effect of EM on body weight gain, dressing percentage,

89   abdominal fat and serum cholesterol content of broilers by supplementing EM in drinking water,

90   and it was found that birds fed with EM had higher weight gains, feed efficiency, while lower

91   feed intake and serum cholesterol content than that in control birds. Besides, EM also has a

92   beneficial effect on promoting animal growth and health. Further studies showed that EM could

93   also improve meat quality, increase slaughter rate, and reduce the rate of death in economic

94   animals (Jagdish et al., 1993; Alvarez et al., 1994; Silva et al., 2000; Patterson et al., 2003;

95   Alagawany et al., 2018; Abd et al., 2020). In contrast, some research results reported that the

96   supplementation of EM in chicken's feed had no significant effect on mortality, feed conversion

97   ratio (FCR) and weight gain (Wondmeneh et al., 2011).

98   Partridge shank chick, a local broiler breed in China, is a relatively smaller body size chick

99   with the features of tender meat and high nutritional value as well as a special flavor when

100   cooked. Therefore, it is very well favored by consumers in China. According to the previous

101   reports, local breeds of broiler chick account for 46.52% of broiler meat in China in 2017, and

102   this proportion was continuously increased in 2018 (Zhao et al., 2019). However, the growth rate

103   of partridge shank broiler chick is slow. This characteristic may attribute to its genetic basis, the

104   environmental factor(s), nutrition, and so on. The gut of animals is important site of nutrient

105   absorption in animals, and better development of the intestinal system could benefit the nutrient

106   absorption and improve animal growth performance and health (Mekbungwan et al., 2004).

107   However, the information regarding the development of intestinal histomorphology and gut

108   microbiota of partridge shank broiler chicks is roughly unknown, and so does the knowledge on

109   the effect of EM on the growth performance of the broilers. We hypothesized that the EM would

110   improve the growth performance and the structure and composition of gut microbiota, perhaps

111   via a mechanism of inhibiting the colonization of bad bacteria. The aim of the study was to

112   evaluate the effects of EM on the growth performance, intestinal gut health and gut microbiota of

113   partridge shank broilers.

114

115   **MATERIALS AND METHODS**

116   **Ethics Statement**

117   All procedures involving live animals were verified and approved by the Office of Animal Care

118   and Use of Jiangxi Agricultural University (protocol number JXAU-LL-20190022). The chicks

119   used in this study were housed at the Animal Research Unit of Jiangxi Agricultural University,

120   located at the college of Animal Science and Technology in Nanchang, Jiangxi, China.

121   **Chicks and experimental design**

122   Ten-day-old female partridge shank broiler chicks (n=100) were purchased from a local

123   commercial hatchery. All of the chicks had been individually wing-tagged, and immunized with

124   the vaccines against Marek's disease, Newcastle disease, and Infectious bursal disease at 1, 4 and

125   10 days old, respectively. The experiment was carried out for a 20-day period starting in Jan,

126   2020. All broiler chicks had ad libitum access to feed and water, and the feed was offered four

127   times daily at 06.00 am, 11.00 am, 16.00 pm, and 20.00 pm, respectively. The chicks were then

128   randomly divided into two experimental groups, and each group included 5 repetitions with 10

129   chicks per replication. All chicks of each replication were housed in 0.96 x 0.96 m chick coops

130   (at the Chicken Experimental Unit, no. 109, Jiangxi Agricultural University, China) under the

131   same environmental conditions, including a constant temperature of 28 to 31℃ and 20 h daily

132   lighting access throughout the experiment.

133   The nutrient levels of the basal diet (maize-soybean-based meal diet) corresponded to the

134   NRC (1994) recommended requirements for broilers (Table 1). The EM suspensions used in this

135   study was generously provided by Prof. Nanhui Chen (Department of Preventive Veterinary

136   Medicine, Jiangxi Agricultural University, China). Chickens in experimental group, designated

137   as EM-treated group (abbreviated as group EM), were fed a basal diet supplemented with 0.5 ml

138   (about 2.5 x 10^9 colony-forming unit) EM per chick/day for 20 days and the chicks in the

139   negative control group, designated as non-EM-treated group (abbreviated as group B) were just

140   fed by the basal diet for 20 days. The bacterial composition of the EM used in this study was

141   determined by 16S rRNA sequencing on Illumina HiSeq 4000 platform, and the bacterial

142   background information of EM used is supplied in Supplementary Table S1. The initial and final

143   weights, daily feed intake of the chicks in each group were recorded, and the feces of five chicks

144   in each group were sampled at the 1st, 10th, and 20th experimental day. Then the feed intake was

145   daily measured, body weight (BW) gain was measured at the end of experiment and then the data

146   were used to calculate average daily intake (ADFI), average daily gain (ADG), and feed/gain

147   ratio (F/G). At the 20th experimental day, 5 chicks of each group were euthanized by

148   pentobarbital sodium and dissected, and the intestinal tissues and cecal contents were collected.

149   For gut microbiota profiling, excreta at the 1st, 10th and 20th experimental day and cecal

150   contents at the 20th experimental day were collected in both groups (designated as EM0, EM10,

151   EM20, and EM20C for samples from EM-treated chicks; and B0, B10, B20, and B20C for

152   samples from non-EM-treated chicks).

153   **Histomorphology observation**

154   At necropsy, different sections of intestines were examined and collected for histological

155   observation according the previous methods in our lab (Zhang *et al*, 2020). For each tissue

156   section, at least ten villi and crypts were measured using the cellSens Standard system (Olympus,

157   Japan) with villous height (VH) and crypt depth (CD), which would be used for the calculation

158   of VH/CD ratio.

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

160   Bacterial genomic DNA were extracted by the QIAamp DNA Stool Mini Kit (Qiagen,

161   Hilden, Germany) and quantified according to the previous method (Song et al., 2017).

162   Amplification of the hypervariable V4 region of 16S rRNA gene was performed by using

163   'universal' primers 515F (5'-GTGYCAGCMGCCGCGTAA-3') and 806R (5'-

164   GGACTACNVGGGTWTCTAA-3') flanked with adapter and barcode sequences (Kuczynski et

165   al., 2011). The PCR was carried out under the following conditions: 95℃ for 5 min; 25 cycles of:

166   95℃ for 30 s, 56℃ for 45 s, 72℃ for 30 s; a final extension of 72℃ for 10 min, and then hold at

167   4℃. The amplicons were cleaned by using AMPure XP beads (Beckman Coulter, Brea, CA,

168   USA), and then normalized, pooled with the adapters and the dual indices using the Nextera XT

169   Index Kit (Cat No.: FC-131-2001, Illumina, San Diego, CA, USA). A second PCR amplification

170   with 5 cycles were executed with Nextera XT Index primers in following conditions: 95℃ for 4

171   min; 5 cycles of: 95℃ for 30 s, 55℃ for 40 s, 72℃ for 40 s; a final extension of 72℃ for 5 min,

172   and then hold at 4℃. The PCR products were cleaned up again with AMPure XP beads, and thus

173   the sequencing libraries were established. The libraries were validated to the expected size of

174   about 440 bp on a Bioanalyzer trace for the final library. The libraries were quantified using a

175   Qubit 4.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) according to the

176   fluorometric quantification method using dsDNA binding dyes. The concentration of each DNA

177   library was determined by an Agilent Technologies 2100 Bioanalyzer. For sequencing, the

178   individual library was diluted for 4 nM, and aliquoted with 5 μl of diluted DNA, then pooled and

179   sequenced on the Illumina Hiseq 4000 platform in paired-end (PE) technology at 2 x 250 nt

180   using Illumina v2 kit (Illumina, San Diego, CA, USA) in Guhe Information Co., Ltd in Hanzhou,

181   China.

182   **Metagenomic analysis**

183   The raw reads from 16S rRNA sequencing were automatically input for quality control,

184   trimming, demultiplexing of samples and then generated fastq output files. Afterwards, the reads

185   were subjected to further proceeding by pipeline QIIME 2 (http://qiime.org/). Operational

186   taxonomic units (OTUs), included de-replication, cluster, detection of chimera, were picked

187   using Vsearch v1.11.1 based on a 97% 16S rRNA gene sequence identity level (Rognes et al.,

188   2016). Taxonomic assignment of individual datasets was determined at several taxonomic levels:

189   kingdom, phylum, class, order, family, genus, and species by using SILVA 128 (Quast et al.,

190   2013). OTUs classified as chloroplasts or mitochondria were subsequently removed. The

191   obtained sequences classified as bacteria and archaea were examined with BLAST (Basic Local

192   Alignment Search Tool) (Mount, 2007).

193   Alpha diversity was calculated with QIIME, including index of observed species, chao1,

194   shannon, simpson, and PD_whole_tree. Beta diversity was determined by using QIIME with the

195   matrix of weighted and unweighted Unifrac distance. LEfSe analysis was performed by using

196   linear discriminant analysis (LDA) to estimate the different size of the effect of abundance of

197   each component (species), and to identify communities or species that had significant differences

198   in the classification of the samples (Segata et al., 2011).

199   **Statistical analysis**

200   The differences of data between EM-treated group and non-EM-treated group were analyzed by

201   student t test in SPSS 26.0 (IBM, USA). The replicate was defined as the experimental unit.

202   Comparisons of parameters of growth performance across the groups were carried out by one-

203   way analysis of variance (ANOVA) and significant differences among group means were

204   determined using the least significant difference (LSD) test. The beta diversity indices were

205   calculated based on the principal co-ordinates analysis (PCoA) method (Quinn Gp, 2002).

206   Kruskal-Walls test was used to identify the difference of alpha diversity indices and bacterial

207   species which showed significant differences between different groups by R stats package. A p-

208   value of <0.05 was set as the statistically significant level.

209

RESULTS

Effects of EM on growth performance of partridge shank broiler chicks

212   In this study, the diet with the addition of EM significantly increased the BW, ADG and

213   decreased the FCR at the 10th ($P < 0.001$) and 20th ($P < 0.05$) day of the experiment when

214   compared with the controls (Table 2). The BW gain for all the evaluated periods (day 1 to 10,

215   day 1 to 20) was improved for chicks supplemented with EM. Similarly, ADG from day 1 to 10,

216   from day 11 to 20 and overall period (day 1 to 20) were increased in EM-treated chicks.

217   Moreover, FCR was decreased during day 1 to 10, and day 11 to 20, while no significance

218   difference in all evaluated period. The EM addition did no significant impact on ADFI.

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

220   Diet with the supplementation of EM significantly increased the jejunal villus height ($P < 0.001$),

221   but decreased the jejunal crypt depth (CD) ($P < 0.001$), and thus increased the ratio of jejunal

222   villus height to crypt depth (VH/CD, $P < 0.001$) but decreased the jejunal crypt depth ($P <$

223   0.001). Furthermore, EM supplementation remarkably increased both ileal ($P < 0.001$) and cecal

224   ($P < 0.001$) villus height and the ratio of VH/CD ($P < 0.001$), but decreased ileal ($P < 0.05$) and

225   cecal ($P < 0.05$) crypt depth (Table 3).

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

227   The samples (n=40) were sequenced on an Illumina HiSeq 4000 platform and a total of

228   3,505,030 raw sequence reads were generated. After quality control, 3,234,992 (92.30%) clean

229   reads were obtained, with an average of 80,874 clean sequences per sample (supplementary

230   Table S2). Shannon, Simpson and Chao1 indices were employed to evaluate the alpha diversity

231   within the sequence datasets based on the observed OTUs. Of the alpha diversity indices, no

232   significant variation was observed between the comparable groups B0/EM0, B10/EM10,

233   B20/EM20, and B20C/EM20C, indicating EM had limited influence on the alpha diversity

234   indices (Table 4). The beta diversity among groups was presented on principal co-ordinates

235   analysis (PCoA) to distinguish the microbial communities (Figure 1). The results revealed that

236   the microbial communities in cecal contents showed a striking distinctness with that in excreta.

237   Clusters of excreta microbiota were superimposed over the PCoA analysis and represented the

238   differences among the groups.

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

240   **EM-treated chicks**

241   In the composition analysis at the phylum level, *Firmicutes* accounted for the most of the relative

242   abundance (63.24%~92.63%), followed by *Proteobacteria* (0.62%~23.94%), *Bacteroidetes*

243   (0.80%~7.85%), *Actinobacteria* (0.06%~13.69%) and others. With increasing age, the

244   abundance of *Firmicutes* tended to decrease and the abundance of *Proteobacteria*, *Bacteroidetes*

245   and *Actinomycete* tended to increase (Figure 2 and Table S3). At the genus level, *Lactobacillus*

246   had the highest relative abundance in the excreta samples (33.26%~78.03%), followed by

247   *Streptococcus* (0.01%~19.07%), *Enterococcus* (0.16%~20.94%), and *Bacteroides*

248   (0.46%~5.27%). Similarly, the abundances of *Lactobacillus* in feces in both EM-treated and non-

249   EM-treated broiler chicks were reduced, while the abundances in EM-treated chicks were higher

250   than that in non-EM-treatedled *chick*s. However, in cecum samples, an unclassified genus from

251   the family *Lachnospiraceae* accounted for the most of the relative abundance (24.02%~36.41%),

252   followed by unclassified genus from the order *Clostridiales* (20.32%~23.40%), unclassified

253   genus in the family *Lachnospiraceae* (6.50%~7.30%), and *Ruminococcus* (2.82%~4.63%)

254   (Figure 3 and Table S4).

255   **Gut microbiota landscape of non-EM-treated chicks**

256   To explore the gut microbiota landscape of the boiler chicks in non-EM-treated group, ANOVA

257   test was performed. At the phylum level, the abundances of *Firmicutes* were significantly

258   decreased (90.53% to 63.24%) from B0 (10d age) to B20 (30d age). While the abundances of

259   *Euryarchaeota*, *Synergistetes*, *Verrucomicrobia*, and *Actinobacteria* were significantly increased

260   as the chicks grew up (Table 5 and Figure S1A). At the genus level, abundances of *Prevotella*,

261   *Coprococcus*, *Desulfovibrio, Gallibacterium*, and *Acinetobacter* tended to be increased from age

262   10d to 30d (Figure S1B).

263   **Gut microbiota landscape of EM-treated chicks**

264   Among the EM-treated partridge shank broiler chicks, four kinds of gut bacteria at the phylum

265   level were significantly different among growth stages of EM0, EM10 and EM20. Bacteria in

266   *Proteobacteria, Synergistetes, and WPS-2* were significantly increased from EM0 to EM20

267   (Table 6 and Figure S2A). At the genus level, the abundances of *Methanobrevibacter*,

268   *Enterococcus*, *Streptococcus,* and *Gallibacterium* were significantly augmented in EM10, while

269   decreased in EM20. The abundances of *Faecalibacterium* and *Megamonas* were reduced during

270   the time of EM supplementation (Figure S2B).

271   **Comparison of excretal and cecal microbiota between EM-treated and non-EM-treated**

272   **chicks**

273   To address the impacts of EM on the structure and abundance of microbiota in feces and cecal

274   contents, the abundances of fecal and cecal bacteria in chicks at the end of the experiment were

275   analyzed. For the excretal microbiota, the abundances of two bacteria *TM7* ($P < 0.01$)and

276   *Tenericutes* ($P < 0.01$)at the phylum level and one at the genus level *Acinetobacte* ($P < 0.05$)

277   were reduced in group EM20 when compared with that in group B20 (Table 7 and Figure S3).

278   As the normal structure of bacterial communities in ceca was very different from that in excreta,

279   the changes of cecal microbiota in EM-treated broiler chicks were different. When compared

280   with the control group, the abundances of *Firmicutes* ($P < 0.001$), *Euryarchaeota* ($P < 0.05$), and

281   *Ruminococcus* ($P < 0.05$) were significantly reduced, while the abundances of *Actinobacteria* ($P < 0.001$)

282   < 0.001) and *WPS-2* ($P < 0.001$) were significantly increased (Table 8 and Figure S4).

283

284

285   **DISCUSSION**

286   As reported from previous studies, probiotics supplements with probiotics in feed could improve

287   the feed intake, weight gain and feed efficiency in broilers (Waititu et al., 2014; Qorbanpour et

288   al., 2018; Jha et al., 2020). Therefore, in order to enhance the growth rate, maintain intestinal

289   integrity, and improve the overall health status of birds in intensive production conditions, the

290   use of probiotic preparations as a supplement is a common practice in poultry production

291   (Wondmeneh et al., 2011). In this study, an EM mixture containing multiple species of bacteria,

292   of which most are naturally existing beneficial microorganisms, including both oxybiotic and

293   anaerobic microbes, was applied to evaluate the effects on the growth performance, intestinal

294   histomorphology, and gut microbiota of partridge shank broiler chicks. Researchers have

295   reported that probiotics had positive effects on BW and ADG of animals (Huang et al., 2019;

296   Tao et al., 2021). The functional inconsistency of probiotics among these studies, including the

297   present study, might attribute to the type and dosage of probiotics being used, and the breeds of

298   the broilers as well.

299   In this study, positive effects of the EM supplementation on BW, ADG and FCR were found

300   in partridge shank broiler chicks. The BW gain and ADG were significantly higher in EM-fed

301   chicks than that in control chicks both at the first phase (0-10th day) and the second phase (11-

302   20th day) of the experiment. However, the ADFI showed no difference between the EM-fed and

303   non-EM-treated control chicks in both phases, which indicated that the EM supplementation

304   could improve the feed conversion efficiency and led to the decrease of FCR. These findings

305   agree with previous studies regarding the beneficial effects of EM and probiotics on the growth

306   performance and gut health of partridge shank broiler chicks (Chantsawang et al., 1999;

307   Safalaoh., 2006; Alkhalf et al., 2010; Xu et al., 2014; Fazelnia et al., 2021). Chantsawang et al.

308 (1999) evaluated the effects of EM supplementation on 4 different types of poultry, and it was

309 found that EM additive could significantly increase breast percentage in Muscovy duck, and

310 decrease ash content of breast meat in Arbor Acers broiler chickens. Safalaoh et al. (2006)

311 demonstrated that the addition of EM in diet significantly increased BW gains (2094±11 g) and

312 ADG compared to broilers on the control diet (2057±15 g) during 1-42 days of age. However, on

313 the other hand, there are some reports which state that probiotics or EM had no role on the

314 growth performance and mortality in broilers. It has been found that the addition of probiotics or

315 prebiotics in broiler diet reduced feed intake (Mokhtari et al., 2010; Chen et al., 2015). Others

316 have demonstrated that the use of probiotics in broiler diet did not affect FCR (Sarangi et al.,

317 2016). In addition, it was found that the weight gain was not affected by supplementation of

318 probiotics (Yousefi et al., 2007). The inconsistent results of aforementioned studies might

319 attribute to multiple factors, including probiotic types and dose being used, and other potential

320 element(s) (Kabir., 2009; Sohail et al., 2012).

321   In this study, the EM addition positively influenced the histomorphological characteristics of

322 the broilers' intestine. Histological observation indicated the supplementation of EM increased

323 the height of intestinal villi in jejunum, ileum and cecum. The structure of intestinal villi were

324 covered with the intestinal epithelium, under which there was a continuous cell layer of

325 myofibroblasts that could regulate the epithelial renewal and defense processes (Ackermann et

326 al., 1974). Furthermore, EM also decreased the intestinal crypt depth (CD) and increased VH/CD

327 ratio of broilers. Crypts are associated with the proliferation of epithelial cells by producing

328 defensins and dendocrine substances (Manning et al., 2004). It has been demonstrated that

329 probiotics *Saccharomyces boulardii* and *Bacillus cereus* had beneficial effects on the epithelial

330 structure and cryptic morphology (Baum et al., 2002). Awad et al. (2009) found that addition of

331 probiotics composed of *Lactobacillus salivarius* and *Lactobacillus reuteri* in feed and found that

332 probiotics significantly increased the BW, average daily weight gain, and improved the villus

333 integrity in small intestines, increased the VH/CD ratio in duodenum in broilers. The EM used in

334 this study contained multiple probiotic bacteria, such as *Lactobacillus* (abundance of 84% ±12%,

335 Table S1) and *Bacillus* (0.09% ± 0.11%), which might benefit the villus and cryptic morphology

336 and then promote the intestinal health.

337   Probiotics were suitable for domestic animals, because they can inhibit the growth of

338 pathogenic bacteria and promote the growth of beneficial bacteria by producing different

339 metabolites and thus improve the gut microecological environment (Cummings and Kong, 2004;

340 Attia et al., 2013; Sun et al., 2019). Similar results were observed in the present study. Although

341 the abundances of *Lactobacillus* were reduced in the chicks as they got older, the abundance of

342 *Lactobacillus* in EM-treated partridge shank broiler chicks were elevated when compared with

343 that in non-EM-treated broiler chicks. The abundance of *Acinetobacter* was significantly lower

344 in EM20 compared to that of B20. It is well known that most of the members of *Acinetobacter*

345 enteropathogenic and can cause infections (Michalopoulos and Falagas, 2010). The commonly

346 encountered pathogenic or zoonotic bacteria affecting birds, including *E. coli*, *Streptococcus*, and

347 *Clostridium* were slightly reduced in the gut of EM-treated partridge shank broiler chicks.

348 Together, the supplementation of EM in feed could ameliorate the community and structure of

349 the intestinal microbiota of partridge shank broiler chicks.

350

CONCLUSIONS

352 In this study, we observed that the feed supplemented with EM could increase the body weight

353 and average daily gain, and reduced feed conversion ratio, enhance intestinal integrity, and

354 balance the gut microflora of partridge shank broiler chicks. The findings might provide an

355 alternative to improve the growth performance and the gut health of partridge shank broiler

356 chicks.

357

358 **ADDITIONAL INFORMATION AND DECLARATIONS**

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364 **Conflicts of Interest**

365 The authors declare that the research was conducted in the absence of any commercial or

366 financial relationships that could be construed as a potential conflict of interest.

367 **Author Contributions**

368 The individual contributions in the present study were as follows: conceptualization and

369 methodology D. S., and Y. T.; validation Z. Y., Z. L., and P. W.; Investigation: Z. Y., B. Z., M.

370 Z., and D. H.; data duration Y. Y., Z. D., G. W., and Q. W.; writing—original draft preparation Z.

371 Y., and Z. L.; project administration D. S., and Q. W.; funding acquisition D. S. All authors have

372 read and agreed to the published version of the manuscript.

373

374 **Data availability statement**

375 All sample raw reads were deposited at the Short Reads Archive (SRA) database belongs to the

376 National Center for Biotechnology Information (NCBI) and are available under Bioproject ID

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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).

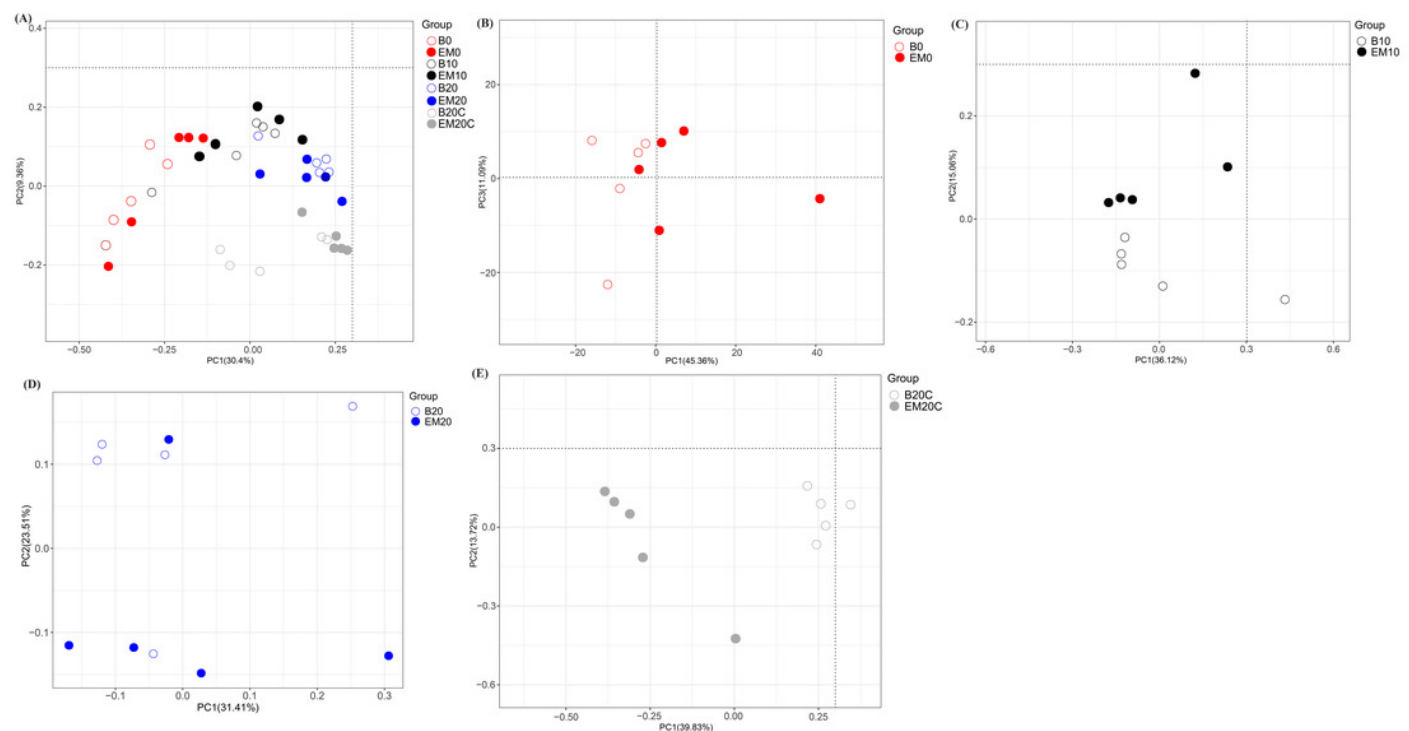



Figure 2

Figure 2. Gut microbial composition at phylum-level.

![Stacked bar chart showing gut microbial composition at phylum level across eight groups: B0, EM0, B10, EM10, B20, EM20, B20C, EM20C. Legend from top to bottom: Unassigned/Others, Fusobacteria, TM7, Euryarchaeota, Tenericutes, Proteobacteria, WPS-2, Synergistetes, Bacteroidetes, Cyanobacteria, Verrucomicrobia, Actinobacteria, Firmicutes.]

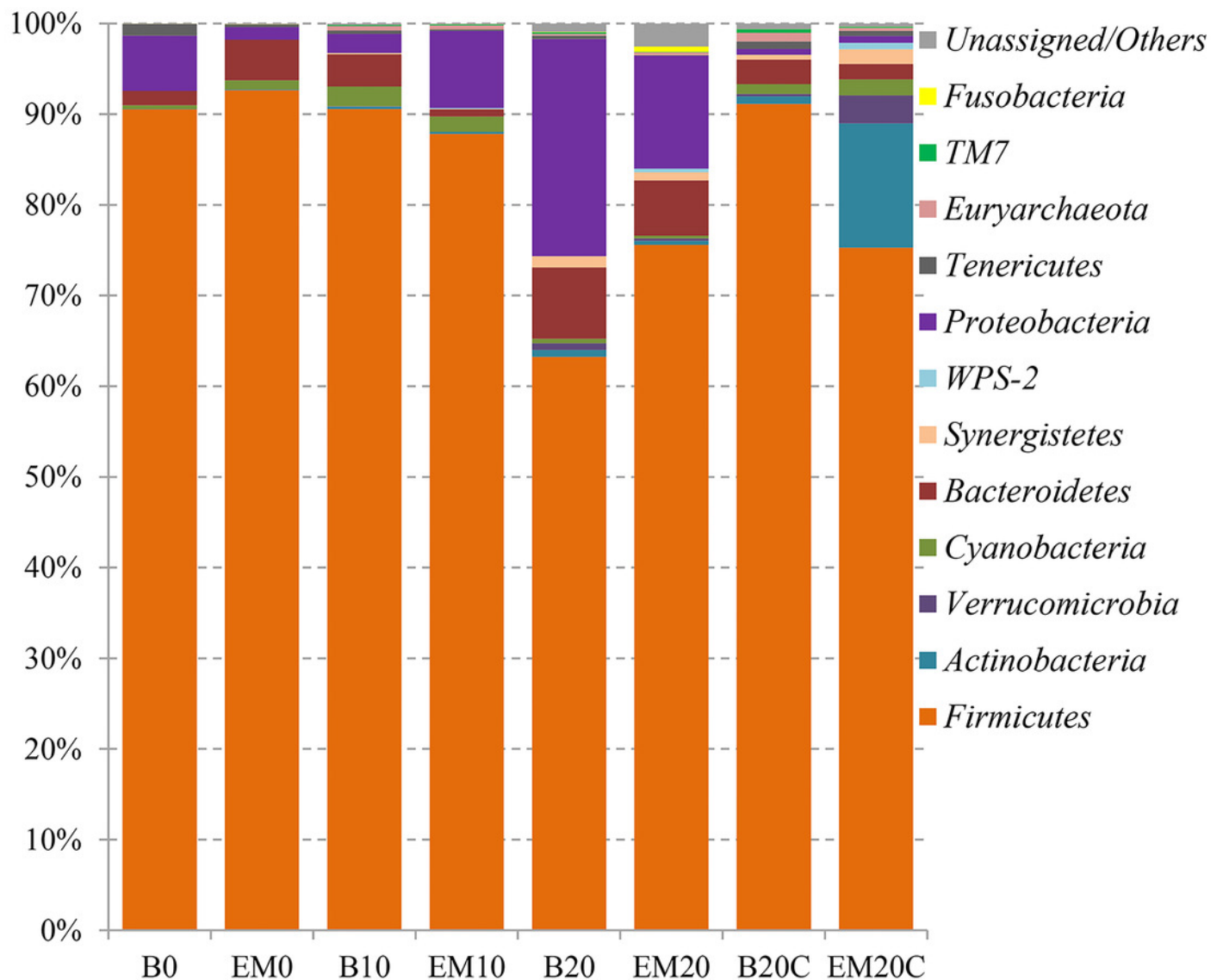
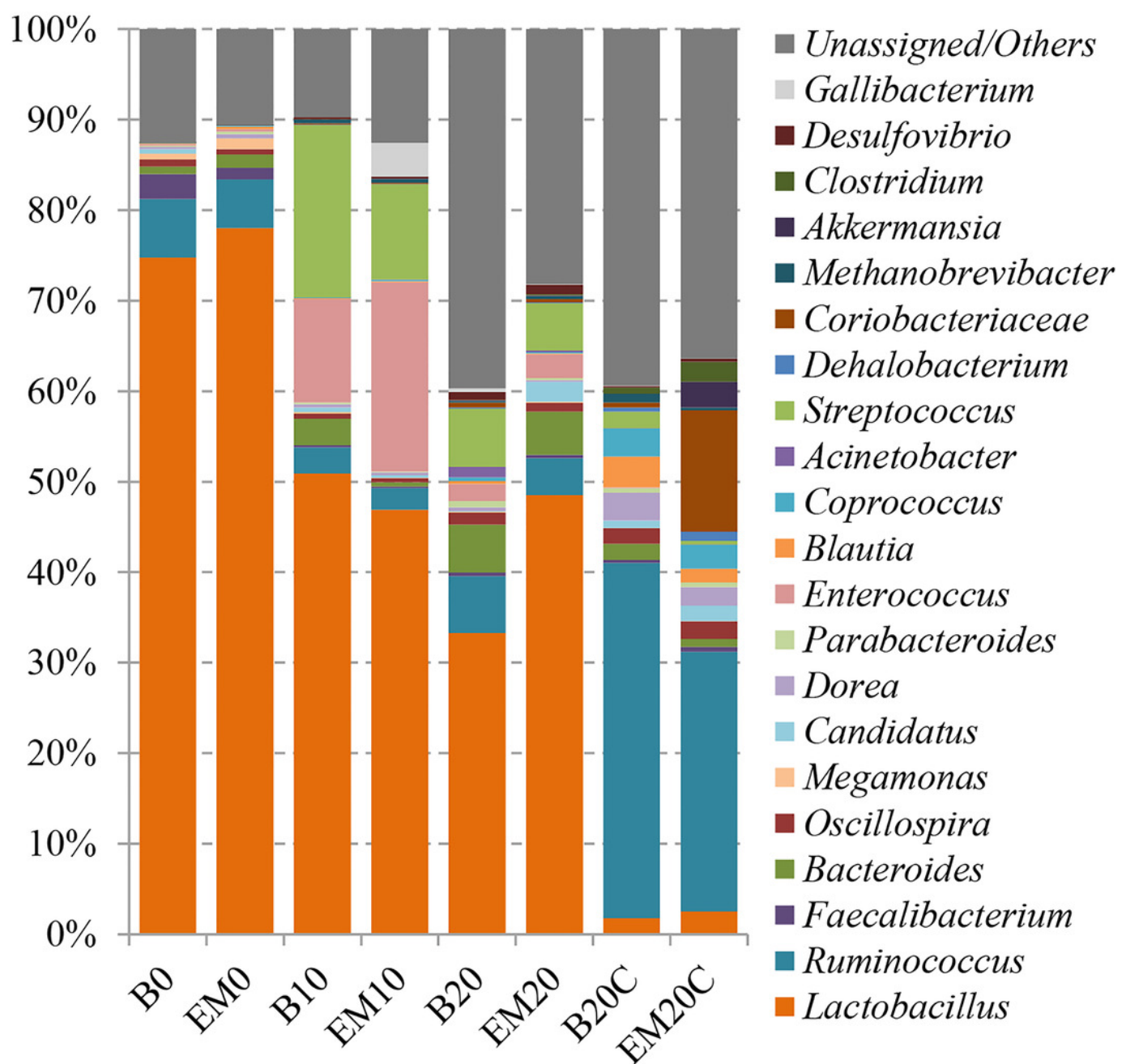



Figure 3

Figure 3. Gut microbial composition at genus-level.

Tables

1 Article

2 **Effects of probiotic supplements on growth performance and**

3 **intestinal microbiota of partridge shank broiler chicks**

4 Yizhe Ye[1,2,†], Zhiquan Li[1,2,†], Ping Wang[1,2], Bin Zhu[3], Min Zhao[1,2], Dongyan Huang[1,2],

5 Yu Ye[1,2], Zhen Ding[1,2], Longrui Li[4], Gen Wan[1,2], Qiong Wu[1,2], Deping Song[1,2,*], Yuxin

6 Tang[1,2]

8 **Tables and Figures**

18 **Tables**

19 **Table 1 Ingredient composition of the basal diet being fed for the broiler chickens used in**

20 **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

21 DM: dry matter; Premix provided per kilogram of diet: vitamin A (all-trans-retinyl acetate), 10,000 IU; vitamin D3 (cholecalciferol), 3,000

22 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

23 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

24 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

25 iodate), 1.1 mg; Se (from sodium selenite), 0.3 mg.

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

29 **with or without EM**

Item	EM-treated boilers	Non-EM treated boilers	p–value (ANOVA)
BW, g			
1 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			
1-10 days	36.49 ± 8.54	35.47 ± 6.51	0.766
11-20 days	56.74 ± 7.58	53.39 ± 7.07	0.321
1-20 days	46.62 ± 13.02	44.43 ± 11.32	0.574
ADG, g/day			
1-10 days	12.86 ± 1.27	10.85 ± 1.07	0.001**
11-20 days	28.91 ± 3.03	27.85 ± 1.85	0.048*
1-20 days	20.89 ± 3.38	19.33 ± 2.04	0.025*
FCR			
1-10 days	2.84	3.27	0.021*
11-20 days	1.96	1.92	0.049*
1-20 days	2.40	2.59	0.053

30 BW=body weight; AVG=average; SD=Standard deviation; ADFI = average daily feed intake; ADG = average daily gain; FCR= feed conversion

31 ratio. * indicates $0.01 < p\ value < 0.05$, ** indicates $0.01 < p\ value < 0.001$.

38

39

40

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

42 **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***

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

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45 **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

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

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	***

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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	***

51 **Table 7 Abundance differences of bacteria in feces between EM-treated and non-EM-**

52 **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	*

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54 **Table 8 Abundance differences of bacteria in cecal contents between EM-treated and non-**

55 **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	*

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67 **Figure Legends**

68 **Figure 1. Principal component analysis (PCoA) based on the sequences from all samples**

69 **tested (A), excreta samples from the 0 (B), 10th (C), and 20th (D) experimental day, and**

70 **cecal content samples from the 20th experimental day (E).**

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

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

73

74 # Supporting Tables

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

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

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

78 **phylum level.**

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

80 **genus level.**

81

82 # Supporting Figures

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

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

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

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

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

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

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

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