Glycerol monolaurate ameliorated intestinal barrier and immunity in broilers by regulating intestinal inflammation, antioxidant balance, and intestinal microbiota

Linglian Kong[a], Zhenhua Wang[b], Chuanpi Xiao[c], Qidong Zhu[a], Zhigang Song[a]#

[a] Department of Animal Science and Technology, Shandong Agricultural University, Taian, Shandong 271018, China

[b] Center for Mitochondria and Healthy Ageing, College of Life Sciences, Yantai University, Yantai, Shandong 264005, China

[c] Precision Livestock and Nutrition Unit, Gembloux Agro-Bio Tech, University of Liège, Gembloux 5030, Belgium

Running Head: GML ameliorated intestinal barrier and immunity

#Address correspondence to Zhigang Song, zhigangs@sdau.edu.cn

ABSTRACT

Extensive interactions occur between a poultry host and its gut microbiome. Glycerol monolaurate (GML) possesses a large range of antimicrobial and immunoregulatory properties. This study was conducted to investigate the impact of different doses of GML (basal diets complemented with 0, 300, 600, 900, or 1200 mg/kg GML) on growth performance, intestinal barrier, and cecal microbiota in broiler chicks. Results revealed that feed intake increased after 900 and 1200 mg/kg GML were administered during the entire 14-day experiment period. Dietary GML decreased crypt depth and increased the villus height-to-crypt depth ratio of the jejunum. In the serum and jejunum, supplementation with more than 600 mg/kg GML reduced interleukin-1β, tumor necrosis factor-α, and malondialdehyde levels and increased the levels of immunoglobulin G, jejunal mucin 2, total antioxidant capacity, and total superoxide dismutase. GML down-regulated jejunal interleukin-1β and interferon-γ expression and increased the mRNA level of zonula occludens 1 and occludin. A reduced expression of toll-like receptor 4 and a tendency of down-regulated nuclear factor kappa-B was shown in GML-treated groups. In addition, GML modulated the composition of the cecal microbiota of the broilers, improved microbial diversity, and increased the abundance of butyrate-producing bacteria. Spearman's correlation analysis revealed that the genera *Barnesiella*, *Coprobacter*, *Lachnospiraceae*, *Faecalibacterium*, *Bacteroides*, *Odoriacter*, and *Parabacteroides* were related to inflammation and intestinal integrity. In conclusion, GML ameliorated intestinal

morphology and barrier function in broiler chicks probably by regulating intestinal immune and antioxidant balance, as well as intestinal microbiota.

IMPORTANCE

Antibiotic residues and resistance issues led to the ban of antibiotic growth promoters. GML is considered an efficacious antibiotic growth promoter alternative for animal health and has the potential to become a unique fungicide owing to its established safety, antibacterial properties, and immunomodulatory capacity. Despite the potential of GML as an additive in poultry feed, little is known about the influence of GML on cecal microbiota in broilers. The significance of our research was to determine the microbial mechanism by which GML worked.

KEYWORDS: glycerol monolaurate, intestinal barrier, antioxidant, inflammation, microbiota

INTRODUCTION

Antibiotics play a significant role in disease prevention and growth promotion in the poultry industry. Despite increasing demand for poultry, antibiotic residues and resistance issues led to the ban of antibiotic growth promoters (1), pressuring the industry to find alternatives for maintaining poultry flock health. One promising approach is immune modulation, in which natural host mechanisms are exploited to enhance and modulate bird's immune response (2). Targeted dietary supplementation or using a feed additive may be useful in the immunomodulation of the immune system. These ingredients can reduce the negative impacts of environmental stressors on animal immune systems and production performance (3). For instance, antimicrobial peptides are feed additives that neutralize lipopolysaccharide (LPS) from *Pseudomonas aeruginosa* at the cellular level and significantly inhibit tumor necrosis factor (TNF-α) and nitric oxide (NO) production in the macrophages of LPS-treated mice (4). Extensive research has been carried out to evaluate an array of products as alternatives to antibiotic growth promoters; such products, including food industry by-products, plant metabolites, non-digestible oligosaccharides, natural by-products, essential minerals, amino acids, medicinal herbs, organic acids, and essential oils, can at least partially alter immune function in poultry (2).

Glycerol monolaurate (GML), a fatty acid composed of glycerol and lauric acid, possesses a large range of antimicrobial and immunoregulatory properties (5). Glycerol monolaurate is considered a food-safe emulsifier endorsed by the Food and Drug Administration and recognized as a nontoxic compound even at relatively high

dose levels (6). Recent studies revealed that GML is an efficacious antibiotic growth promoter alternative for animal health (7) and has the potential to become a unique fungicide owing to its established safety, antibacterial properties, and immunomodulatory capacity (8). Glycerol monolaurate with a supplementary dose of up to 5 g/kg to basal diets can enhance the immune status and intestinal histomorphology of broilers (3). In vivo, growth performance and intestinal development are improved by dietary GML in mice and laying hens through intestinal microbiota alteration (6).

Despite the potential of GML as an additive in poultry feed (9), little is known on the influence of GML on intestinal barrier and cecal microbiota in broilers. Extensive interactions occur between a poultry host and its gut microbiome, particularly during exchange of nutrients and modulation of host gut morphology, physiology, and immunity (10). We hypothesized that GML can improve immunity, growth performance, and health status by altering gut microbiota. Therefore, the present study was designed to investigate the effects of different doses of GML on performance, immunity, and antioxidant capacity, as well as intestinal barrier and microbiota in broilers.

RESULTS

Growth performance. As shown in Table 3, FI rates increased in the 600, 900, and 1200 mg/kg GML-treated groups compared with those in the 300 mg/kg group ($P < 0.05$) after 7–14 days of treatment but showed no significant difference from those

of the control group ($P > 0.05$). However, the administration of 900 and 1200 mg/kg GML increased FI compared to the control and 300 mg/kg GML-treated group during the overall period ($P < 0.05$). Dietary GML did not affect the BW, BWG, and FCR of broiler chicks. ($P > 0.05$).

Intestinal morphology analysis. As shown in Table 4, GML decreased CD ($P < 0.05$) and increased VCR ($P < 0.05$) in the jejunum on day 7 and 14 with increasing dose (600, 900, and 1200 mg/kg). No effect was observed on jejunal VH after dietary treatment with GML ($P > 0.05$).

Serum and intestinal biochemical indicators. On days 7 and 14, dietary treatment with 600, 900, and 1200 mg/kg GML reduced serum IL-1β level ($P < 0.05$) compared with the level in the control group (Table 5). A reduction ($P < 0.05$) of serum TNF-α level was recorded in 7-day-old broilers treated with 900 and 1200 mg/kg GML. In addition, 1200 mg/kg GML increased serum IgG and jejunal mucin 2 levels relative to those of the control on day 14.

Antioxidant capacity in serum and jejunum. As shown in Table 6, dietary GML reduced MDA content in the serum and jejunum on 14-day-old broilers ($P < 0.05$). Serum SOD level increased in broilers fed with 1200 mg/kg GML on day 7 ($P < 0.05$) relative to that of the control and tended to improve 14 days after GML supplementation ($P < 0.1$). In the jejuna of 7-day-old broilers, dietary treatment with GML increased SOD levels ($P < 0.05$), and the highest level was found in the 1200 mg/kg group ($P < 0.05$). A higher T-AOC level was observed in the jejuna of the 600 and 1200 mg/kg groups on day 7 ($P < 0.05$) and in the sera after 900 and 1200 mg/kg

GML addition on day 14 ($P < 0.05$).

Relative mRNA expression of jejunal genes. On 7-day-old broilers, dietary treatment with 1200 mg/kg GML down-regulated ($P < 0.05$) jejunal *IL-1β* and *interferon (IFN-γ)* expression compared with that in the control (Table 7). Moreover, 600 mg/kg GML-treated broilers showed higher *zonula occludens (ZO)-1* and *occludin* expression levels than the control ($P < 0.05$). The mRNA level of *occludin* increased in the jejunum with 1200 mg/kg GML supplementation ($P < 0.05$). On 14-day-old broilers, *IL-1β* expression decreased in the 1200 mg/kg group ($P < 0.05$), and *IFN-γ* expression decreased in the 600, 900, and 1200 mg/kg groups ($P < 0.05$). Jejunal *occludin* expression was not altered by GML supplementation, compared with that in the control ($P > 0.05$), but difference in jejunal *occludin* expression between GML-treated groups was observed ($P < 0.05$). The expression of toll-like receptor4 (TLR4) was decreased in 600 and 1200 mg/kg GML-treated broilers ($P < 0.05$). In addition, dietary GML tended to reduce the expression of jejunal nuclear factor kappa-B (NF-κB) in 14-day-old broilers ($P < 0.1$).

Composition and community diversity of cecal microbiota. On 7-day-old broilers, 378 OTUs were found among the five groups, and 626, 682, 843, 818, and 827 specific OTUs were unique to the control, 300, 600, 900, and 1200 mg/kg GML-treated groups, respectively (Fig. 1A). On 14 days of age, 938, 845, 616, 964, and 973 specific OTUs existed respectively in the Con, 300, 600, 900, and 1200 mg/kg GML-treated groups (Fig. 1A). As shown in Table 8, diets supplemented with 600, 900, and 1200 mg/kg GML increased Chao 1 and observed indices in 7-day-old

broilers compared with the control ($P < 0.05$). However, no significant differences between the control and GML-treated groups were observed for alpha diversity on 14-day-old broilers ($P > 0.05$). Broilers fed with 1200 mg/kg GML had the highest ($P < 0.05$) Chao 1, PD, observed, Shannon, and Simpson indices among the GML-treated groups. On day 7, unweighted UniFrac-based nonmetric multidimensional scaling analysis indicated no obvious difference ($P > 0.05$) in the β diversity of cecal microbiota between each group (Fig. 1B). In the 14-day-old broilers, the microbiomes in the 900 and 1200 mg/kg GML groups were completely separated from the control and 300 mg/kg on the NMDS2 axes ($P = 0.003$; Fig. 1B). PERMANOVA analysis based on unweighted UniFrac distance revealed that the cecal microbiota in the 600, 900, and 1200 mg/kg GML-treated groups had a higher β diversity index than that in the control on day 7 ($P < 0.05$) and in the 900 and 1200 mg/kg GML-treated groups on day 14 ($P < 0.05$; Fig. 1C).

The relative abundance of community at the phylum level is shown in Fig. 2A. At the phylum level, the *Firmicutes/Bacteroidetes* ratio decreased in the ceca of 7-day-old broilers after 600 and 1200 mg/kg GML supplementation ($P < 0.05$) but was not altered in each group on day 14 ($P > 0.05$; Fig. 2B). Supplementation with 600 and 1200 mg/kg GML increased the amount of *Bacteroidetes* relative to the amounts in the control, 300, and 900 mg/kg groups ($P < 0.05$; Fig. 2C). The relative abundance of *Actinobacteria* decreased in the ceca of the 14-day-old broilers that received 600 and 900 mg/kg GML ($P < 0.05$; Fig. 2C). At the genus level, the 300 mg/kg GML-treated group was enriched with *Barnesiella* and *CHKCI001* on days 7

and 14 ($P < 0.05$), respectively (Fig. 3B). The proportion of *Coprobacter* and reduced *Lachnospiraceae_FE2018_group* (Fig. 3B) increased in the 600 mg/kg GML-treated group ($P < 0.05$). The relative abundances of *Barnesiella*, *Odoribacter* and *Parabacteroides* increased ($P < 0.05$) in the broilers fed with 900 mg/kg GML (Fig. 3B). In addition, except the decreased *Lachnospiraceae_FE2018_group*, 1200 mg/kg GML significantly increased the abundances of *Barnesiella*, *Faecalibacterium*, *Bacteroides*, *Odoribacter*, and *Parabacteroides* and *CHKC001* level in the ceca of the broilers ($P < 0.05$) relative to those in the control and broilers that received other GML doses (Fig. 3B).

The specific bacterial taxa associated with GML treatment was identified through linear discriminant analysis effect size (LEfSe, LDA score > 4) analysis. As shown in Fig. 4A, only the genus *Blautia* was observed to be significantly abundant in the 300 mg/kg group. The 600 mg/kg GML-treated group showed the enrichment of the genus *Coprobacter,* family *Bacteroidetes*, phylum Bacteroidia, and order Bacteroidales. The family *Lactobacillaceae* and genus *Lactobacillus* predominated in the 900 mg/kg GML group. Furthermore, the genera *Faecalibacterium*, *Barnesiella*, and *Intestinimonas* were enriched in the ceca of 1200 mg/kg GML-treated broilers on day 7. At the 14th day of treatment, the abundance of genus *UBA1819* increased in the 300 mg/kg GML-treated group (Fig. 4B). LEFse analysis indicated significant distinctive bacteria of family *Barnesiellaceae* and genus *Barnesiella* in the 600 mg/kg GML-treated group (Fig. 4B). Genus *Arthromitus* and *CHKCI001* were overrepresented in the 900 mg/kg group (Fig. 4B). The cecal microbiota in the 1200

183 mg/kg GML-fed group was characterized by phylum, family *Lachnospiraceae*, and
184 genus *Coprobacter* (Fig. 4B). Through Spearman correlation analysis, the
185 relationships of changes in intestinal microflora at the genus level with intestinal
186 integrity, inflammatory factors, antioxidant enzymes, tight junction proteins, and
187 TLR/NF-κB signal pathway were discussed (Fig. 4C). The levels of inflammatory
188 factors were positively associated with the genera *Lachnospiraceae_UCG_008*,
189 *Erysipelatoclostridium*, *Unspecified_Bacteria*, *Anaerostipes*,
190 *Eubacterium_hallii_group*, *Eisenbergiell*a, *Lachnospiraceae_UCG_010*, and
191 *Anaerotruncus* but negatively correlated with *Barnesiella*, *Faecalibacterium*,
192 *Coprobacter*, *Odoribacter*, *Parabacteroides*, *CHKCI001*, and *Bilophila*. Intestinal
193 integrity was possibly correlated with the genera *Coprobacter*,
194 *Ruminococcaceae_UCG_010*, *Faecalibacterium*, *Barnesiella*, *Desulfovibrio*,
195 *Bilophila*, *Parabacteroides*, *Phascolarctobacterium*, and *CHKCI001*. The abundances
196 of *Lachnospiraceae_FE2018_group*, *Eubacterium_hallii_group*,
197 *Ruminiclostridium_5*, *Enterococcus*, *Romboutsia*, and Caproiciproducens were
198 negatively associated with intestinal barrier.
199
200 **Discussion**
201 Medium-chain fatty acids (MCFA) have received widespread attention as feed
202 additives, showing positive benefits and improving animal health, production, and
203 feed digestibility (11, 12). Current evidence supports that MCFA and monoglycerides
204 are generally effective in supporting growth performance and intestinal health (13).

Feed intake is the basic feature that guides the growth rates of broilers (14). In the current study, dietary treatment with GML effectively increased FI rates of broiler chicks, consistent with previous findings (9, 15). A combination of gut inflammation, immune cell infiltration, and increased levels of proinflammatory cytokines often reduce FI and cause diarrhea (16). Decreased level of inflammatory factors after GML treatment may be one of the reasons for the increased FI in the present study. However, none of the supplemented treatment groups showed changes in BW, BWG, and FCR, consistent with the previous findings that GML did not alter the growth performance of broilers in the first 28 days of treatment (17). In addition, no effects were reported on the BW, BWG, and FCR after dietary supplementation with four GML levels (0, 1, 3, or 5 g/kg) during all experimental periods (3). Conversely, increased ADFI and BW and reduced FCR were observed in the 28–56-day-old yellow-feathered broilers with dietary GML (18). Broilers fed with GML had increased BWG, ADG, and feed consumption and decreased FCR (9). The positive effect of MCFA on digestibility and growth performance is the inhibition of pathogen proliferation (19).

Diets complemented with the glycerol-esters of MCFAs exert immunomodulatory effects (20). In the current study, reduced IL-1β and TNF-α and increased IgG levels were observed in the sera after dietary treatment with GML. Proinflammatory cytokines IL-6 and TNF-β levels decreased in the sera after GML supplementation, which alleviates systemic inflammatory response in high-fat-diet-fed mice (6). In addition, GML may be considered a topical anti-inflammatory agent (21). It down-regulated the gene expression of jejunal *IL-1β*,

227 *IL-6*, and *IFN-γ* in the present study. Owing to the relatively stable state and long
228 residence time in the gastrointestinal tract, an anti-inflammatory environment was
229 induced instead of systemic inflammation after the administration of a high dose of
230 GML (22). A similar result was observed in this study. The 1200 mg/kg GML dose
231 showed better effects on broiler inflammation and immunity. Monoglycerides and
232 MCFAs exhibit antimicrobial and immunomodulatory activities as additive candidates,
233 thereby mitigating feed pathogen proliferation and improving enteric health in weaned
234 pigs (13). An in vitro study showed that lauric acid treatment reduces the
235 concentration of proinflammatory cytokines (TNF-α, IL-6, and IL-1β) in the culture
236 supernatant of microglia attacked by LPS (23). T cell receptor (TCR)–induced
237 signaling and T cell activation were suppressed by GML, which inhibits T
238 cell-mediated cytokine storm (24). In addition, GML disrupted the lipid dynamics of
239 human T cells, potentially reducing the TCR-induced production of cytokines. This
240 function suggests that GML has an immunomodulatory role (5). The secretion of
241 MIP-3 and IL-8 induced by HIV-1 was inhibited. This result supported the hypothesis
242 that GML has an immunomodulatory effect during infection (8).

243 One of the central ways feed compounds affect immunity is activating NF-κB,
244 which is an inducible central regulator of inflammatory responses involved in most
245 innate immune receptor signaling pathways (25). In this study, GML supplementation
246 tended to decrease jejunal *NF-κB* expression. Lauric acid and GML have minimal
247 impacts on NF-κB activation in the absence of an LPS challenge, although a
248 statistically significant increase can be observed at certain concentrations (25). Such

249 increase partially explains the results of the present study. The canonical pathway of
250 NF-κB activation involves signaling by pattern recognition receptors, such as TLR
251 (26). Our data indicated that dietary treatment with 600 and 1200 mg/kg GML
252 effectively reduced the relative mRNA expression of *TLR4*. Similar results were
253 obtained in a mice experiment in which GML down-regulated *TLR2* MyD88
254 expression in the liver, reducing systemic inflammation in high-fat-diet fed mice (6).
255 Lauric acid increases the activity of NF-κB through the dimerization of TLR2 and
256 TLR1 or TLR6 and the activation of TLR4 (27).

257 Intestinal integrity is a key factor for preventing the invasion of pathogenic
258 microorganisms in broilers (28). Dietary GML tends to increase the VH and VCR and
259 decrease the CD of the jejunum, thus improving growth performance (18). In this
260 study, dietary GML decreased CD and increased VCR in the jejuna of broilers with
261 increasing dose. High VCR is widely regarded as a good indicator of mucosal
262 turnover and is related to strong digestion and absorption capacity (28). VH and VCR
263 in the duodena, jejuna, and ilea of broilers increased after 150 mg/kg GML
264 supplementation, and this increase was considered the reason for the high metabolic
265 rate of feed nutrients and decreased FCR (29). Natural extracts with
266 anti-inflammatory effects restore the damaged intestinal morphology of broilers
267 attacked by LPS (30). Therefore, the improvement in intestinal integrity can be
268 partially explained by the decreased expression of proinflammatory cytokines and
269 down-regulation of *TLR4/NF--κB* signal transduction. After GML was given to the
270 mice, the normal expression levels of *ZO-1*, *occludin*, *claudin-1*, *jam-1*, and *muc 2*

271 suggested that GML maintained the mucosal barrier and intestinal health (22). In this
272 study, dietary GML effectively benefited the jejunal muc 2 content and up-regulated
273 *ZO-1* and *occludin* expression, demonstrating the beneficial effect of GML on
274 intestinal barrier of broilers. The TNF-α and IFN-γ are related to the reduction in
275 epithelial barrier function and increases the permeability of the mucosal barrier (31).
276 The reduction in serum TNF-α level and decrease in jejunal *IFN-γ* expression in this
277 study supported the idea that GML mediates intestinal barrier function through a
278 mechanism associated with the attenuation of intestinal inflammation.

279 Oxidative stress is an important factor for the destruction of mucosal barrier
280 function (32). In the current study, reduced MDA content and increased T-SOD and
281 T-AOC activity in the sera and jejuna indicated that GML reduced lipid peroxidation
282 and improved the antioxidant capacity of the broilers. This result is in a line with a
283 previous study (33). In laying hens, increase in SOD level and reduced glutathione
284 and MDA levels were observed, suggesting that GML can decrease lipid peroxidation
285 and enhance the antioxidant capacities of broilers. These features are beneficial to
286 growth performance (17). The peroxidation level in the meat of GML-fed chickens
287 was reduced and proportional to the increase in dietary additive concentration (9).
288 Significant connections between inflammation and oxidative stress have been found.
289 These processes induce each other reciprocally, thereby establishing a vicious cycle
290 that perpetuates and propagates inflammatory response (34). In addition, the
291 proinflammatory signaling cascades triggered by TLR engagement enhance the
292 expression of iNOS, implying that TLR activation may result in oxidative stress (35).

293 Therefore, the improvement in antioxidant capacity in the GML-treated broilers may
294 be related to lowered inflammatory response and down-regulated TLR4/NF--κB
295 pathway. Reduced oxidative stress was beneficial for relieving inflammation.

296 Intestinal microbiota contributes to the maintenance of intestinal physiological
297 structure and function, which are considered relevant to intestinal inflammation,
298 barrier function, and growth performance of a host (36). Our results showed that
299 dietary GML modulated the microbial composition in the ceca of the broilers. A rich
300 community of species enhances the stability of the intestinal microecology and may
301 be related to reduced sensitivity to bacterial invasion and intestinal inflammation (37).
302 Therefore, the alleviated intestinal inflammation of broilers may be associated with
303 the modulated structure and increased diversity of intestinal microbial community
304 structure after GML addition. At the phylum level, GML mainly altered the relative
305 frequency of *Bacteroidetes* and *Actinobacteria* in the ceca of the broilers. Increase in
306 *Actinobacteria* and *Firmicutes*/*Bacteroidetes* ratio is the pattern of an impaired
307 intestinal barrier (38). In this study, the cecal microbiota in GML-treated broilers was
308 characterized by decreased level of *Actinobacteria* and *Firmicutes*/*Bacteroidetes* ratio,
309 which may be the potential reasons for the improved intestinal barrier. In addition,
310 1200 mg/kg GML increased the amount of cecal *Bacteroidetes*, which mainly
311 contributes to the fermentation of indigestible carbohydrates to butyrate (39) and
312 exerts beneficial effects on mucosal barrier integrity through its anti-inflammatory
313 effects (40). At the genus level, the relative abundance of *Barnesiella*, *Coprobacter*,
314 *Lachnospiraceae*, *Faecalibacterium*, *Bacteroides*, *Odoriacter*, *Parabacteroides*, and

315 *CHKCI001* were higher in the GML-treated groups, especially under the dose of 1200
316 mg/kg. Mice fed with 400 and 800 mg/kg GML had higher abundances of *Barnesiella*,
317 which was considered to be positively associated with a healthy state (22).
318 *Lachnospiraceae* can promote health by producing host nutrients and providing an
319 energy supply to the colonic epithelium, as well as maintaining host immune
320 homeostasis (41). *Parabacteroides* are associated with T-cell differentiation by
321 enhancing and maintaining the IL-10-producing Treg cells (42). *Barnesiella*,
322 *Faecalibacterium*, *Bacteroides*, *Odoribacter*, and *Parabacteroides* were positively
323 correlated with the level of SCFAs and produced butyrate, which exerted
324 immunomodulatory and anti-inflammatory effects by mediating the homeostasis of
325 colonic regulatory T cell populations and promoted intestinal integrity (43). In
326 addition, butyrate with antioxidant properties modulates inflammatory response by
327 inhibiting NF-κB and provides energy to the intestinal epithelial cells (38). A
328 significant impact was reported on host health and physiology after GML
329 supplementation, which directly acts on the intestinal microbiota and considerably
330 affects metabolism and immunity (44). Therefore, the increased abundance of
331 butyrate-producing bacteria after GML addition was associated with alleviated
332 intestinal inflammation and improved intestinal morphology of the broilers.

333 In conclusion, dietary GML ameliorated intestinal morphology and barrier
334 function of broiler chicks by ameliorating inflammation and promoting antioxidant
335 status. Our results confirmed the immunomodulatory, antioxidant, and
336 anti-inflammatory properties of GML, which may be associated with the suppression

of the TLR4/NF-κB signaling pathway. The altered structure of the cecal microbiota manipulated by GML may be the main reason for the promotion of intestinal health in the broilers.

Material and methods

Animals, experimental design and management. All experimental procedures were approved by the Ethics Committee of the Shandong Agricultural University and carried out according to the Guidelines for Experimental Animals of the Ministry of Science and Technology (Beijing, People's Republic of China). All feeding and euthanasia procedures were performed with full consideration of animal welfare. A total of 360 one-day-old broilers (Arbor Acres) with an average weight of 45.7 g were randomly divided into five groups as follows: basal diet (control) and basal diets supplemented with 300, 600, 900, or 1200 mg/kg GML, which was purchased from Henan Zhengtong Food Technology Co., Ltd (Henan, China) with purity of more than 90%. The additive dosage of GML was optimized according to previous studies (6, 45, 46). Each group contained six replicates (cage) of 12 broilers per cage. The ingredients and nutrients levels in the basal diet were formulated according to standards of the National Research Council 2012 (Table 1). All broilers were weighed and randomly assigned to 30 metal cages (70 cm × 70 cm × 40 cm), which were equipped with feeders and nipple drinkers. Broilers with similar initial weights were reared in an environmentally controlled room. The temperature was 35 °C initially, and then gradually decreased to 28 °C until the end of the 14-day experiment period.

In the first 3 days, average relative humidity was maintained at approximately 70%, and thereafter maintained between 55% and 65%. In the first week, the broilers were kept under 23 h of light and 1 h of darkness, which were then gradually reduced to 20 and 4 h, respectively.

Growth performance. On days 7 and 14, feed consumption in each replicate and body weight were recorded. Body weight gain (BWG) and feed intake (FI) were calculated subsequently. Spilled feed was carefully collected and weighed for the correction of the final FI data. Feed conversion rate (FCR) was defined as FI:BWG. The data of mortality were recorded and included in the FCR calculation.

Sampling. Two broilers per replicate were randomly selected for sampling after growth performance was determined on 7 and 14 days of age. Blood samples were collected from wing veins and negotiated to glass tubes without anticoagulants, then centrifuged at 3000 rpm for 10 min at 4 °C. Serum was obtained and stored at −20 °C for biochemical analysis. Broilers were slaughtered by cervical dislocation after blood samples were obtained. Approximately 2 cm segments were excised from the jejunum (from the entry point of the bile duct to the Meckel's diverticulum), flushed repeatedly with cold saline solution, and immediately immersed in 4% paraformaldehyde solution for histological examination. Tissue samples (1–2 g) were collected from the jejunum, rapidly frozen in liquid nitrogen, and stored at −80 °C for molecular analysis. The cecum was collected on ice, frozen quickly in liquid nitrogen, transported to the laboratory in a dry-ice bag, and then stored at −80 °C for further microbial analysis.

Jejunal morphology analysis. Jejunum segments were fixed in 4%

paraformaldehyde solution for 24 h, dehydrated, and embedded in paraffin. Tissue sections with 5 μm thickness were cut using a microtome (Leica RM2235, Leica Biosystems Inc., Buffalo Grove, USA), fixed on slides, and stained with hematoxylin and eosin. The images of the jejunum were analyzed with ImageJ analysis software (Version 1.47, Bethesda, MD, USA). Ten intact villi were selected randomly from each section for morphology measurement. Villus height (VH) was gauged from the tip of the villus to the villus–crypt junction. Crypt depth (CD) was defined as the depth of the invagination between adjacent villi. Villus height-to-crypt depth ratio (VCR) was calculated. The mean value of ten values attributed to individual broilers was used in statistical analysis.

Biochemical assay of serum and jejunum. Immune response status in the sera was estimated by detecting the levels of interleukin 1 beta (IL-1β), interleukin 6 (IL-6), tumor necrosis factor-alpha (TNF-α), and immunoglobulin G (IgG) with ELISA kits (MLBIO Co., Ltd., Shanghai, China). All determination procedures were performed strictly according to the manufacturer's instructions. The inter- and intra-assay coefficients of variation (CVs) were less than 10%. The jejunal samples were weighed accurately (0.3 g), homogenized with 2.7 ml phosphate-buffered saline in a weight (g):volume (ml) ratio of 1: 9. The homogenates were centrifuged at 1000 *g* for 10 min at 4 °C, and the supernatants were collected for the detection of secreted immunoglobulin A (sIgA) and mucin 2 levels with ELISA kits (MLBIO Co., Ltd., Shanghai, China). The results were expressed as pg/mg of protein.

Antioxidant assay of serum and jejunum. Malondialdehyde (MDA) levels,

403 total antioxidant capacity (T-AOC), and total superoxide dismutase (T-SOD) activity
404 were measured in each serum and jejunal homogenate with diagnostic kits
405 (intra-assay CV < 5%; inter-assay CV < 8%) purchased from Nanjing Jiancheng
406 Biotechnology Institute (Nanjing, China) according to the manufacturer's instructions.
407 The results were normalized to protein concentration in each jejunal homogenate.

408 **RNA isolation and real-time quantitative PCR.** The total RNA of the jejunum
409 was isolated using Trizol reagent (Invitrogen Biotechnology Inc., CA, USA). RNA
410 quality was evaluated through 1% agarose gel electrophoresis. The reverse
411 transcription of 1 μg of total RNA was performed using PrimeScript® RT reagent kit
412 with gDNA Eraser (RR047A, Takara Bio Inc., Dalian, China). The gene expression
413 was determined through RT-PCR. TB Green Premix Ex Taq (RR820A, Takara Bio
414 Inc., Dalian, China) and ABI 7500 real-time PCR systems (Applied Biosystems, CA,
415 USA) were used. The reaction program was as follows: predenaturation at 95 °C for
416 10 s, then denaturation at 95 °C for 5 s for a total of 40 cycles, and finally annealing
417 and extension at 60 °C for 40 s. Each reaction was repeated three times, and the
418 primer sequences are shown in Table 2. The amplification efficiency of the primers
419 was calculated with a standard curve. The specificity of the amplified products was
420 verified with the melting curve. The relative expression of the target gene was
421 analyzed through the $2^{-\Delta\Delta Ct}$ method after normalization against the geometric mean of
422 the expression of β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and
423 TATA-binding protein (TBP).

424 **16S rRNA sequencing and analysis.** Total DNA was extracted from cecal

contents with an E.Z.N.A.® Soil DNA kit (Omega Bio-Tek, Norcross, GA, U.S.) according to the manufacturer's instructions. DNA purity and concentration were evaluated with a Nano Drop2000 spectrophotometer (Thermo Scientific, Wilmington, USA), and DNA integrity was detected through 1% agarose gel electrophoresis. Bacterial 16S rRNA gene spanning the V3-V4 hypervariable regions were amplified with primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) with a PCR system. PCR reactions were performed in triplicate with a 20 μl mixture consisting of 4 μl of 5 × FastPfu Buffer, 2 μl of 2.5 mM dNTPs, 0.8 μl of each primer (5 μM), 0.4 μl of FastPfu polymerase, and 10 ng of template DNA. The amplification programs were set in ABI GeneAmp® 9700 system (ABI, USA) as follows: 3 min at 95 °C, 27 cycles of 30 s at 95 °C, 55 °C for 30 s, and 72 °C for 45 s, and 72 °C for 10 min. The PCR products were detected through 2% agarose gel electrophoresis and purified with an AxyPrep DNA gel recovery kit (Axygen Biosciences, Union City, CA, USA) and then quantified with a QuantiFluor™-ST blue fluorescence quantitative system (Promega, USA). Purified amplicons were pooled in equimolar amounts, and their paired-end reads were sequenced on an Illumina MiSeq PE300 platform (Illumina, San Diego, USA) by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). Raw fastq files obtained through MiSeq sequencing were demultiplexed, quality-filtered, trimmed, de-noised with trimmomatic, and merged according to the overlapping relationship by FLASH (version 1.2.11, https://ccb.jhu.edu/software/FLASH/index.shtml). The filtered reads were clustered into operational taxonomic units (OTUs) with a 97 %

447 sequence identity with UPARSE (version 7.1, http://www.drive5.com/uparse/).
448 Chimera was removed during clustering. The OTU representative sequence was
449 analyzed with the RDP Classifier (version 2.2,
450 http://sourceforge.net/projects/rdp-classifier/) against the Silva 16S rRNA database
451 (release119, http://www.arb-silva.de) at a confidence threshold of 70%.

452 **Statistical analysis.** Data were presented as mean ± SD. All data were checked
453 for normality with Shapiro–Wilk test (95% confidence level). Statistical differences
454 between groups were analyzed with one-way ANOVA with Tukey's multiple
455 comparisons or by the non-parametric factorial Kruskal-Wallis test. SPSS software
456 (SPSS 26.0, SPSS, Chicago, USA) was used. Differences were considered
457 significantly different at $P < 0.05$. Probability $0.05 < P < 0.1$ were defined as
458 tendencies.

459

460 **ACKNOWLEDGMENT**

461 This work was supported by the Natural Science Foundation of Shandong Province
462 (ZR2020MC170), the National Key R&D Program of China (2018YFD0501401-3),
463 and the Shandong Province Agricultural Industry Technology (SDAIT-11-08).

464

465 **REFERENCE**

466 1. Sánchez-Cordón PJ, Montoya M, Reis AL, Dixon LK. 2018. African swine fever: A
467 re-emerging viral disease threatening the global pig industry. Veterinary journal (London,
468 England : 1997) 233:41-48.

2. Kogut MH. 2017. Issues and consequences of using nutrition to modulate the avian immune response. Journal of Applied Poultry Research 26:605-612.

3. Amer SA, A-Nasser A, Al-Khalaifah HS, AlSadek DMM, Abdel Fattah DM, Roushdy EM, Sherief WRIA, Farag MFM, Altohamy DE, Abdel-Wareth AAA, Metwally AE. 2020. Effect of Dietary Medium-Chain α-Monoglycerides on the Growth Performance, Intestinal Histomorphology, Amino Acid Digestibility, and Broiler Chickens' Blood Biochemical Parameters. Animals : an open access journal from MDPI 11.

4. Wang C, Shao C, Fang Y, Wang J, Dong N, Shan A. 2021. Binding loop of sunflower trypsin inhibitor 1 serves as a design motif for proteolysis-resistant antimicrobial peptides. Acta Biomater 124:254-269.

5. Zhang MS, Sandouk A, Houtman JCD. 2016. Glycerol Monolaurate (GML) inhibits human T cell signaling and function by disrupting lipid dynamics. Scientific reports 6:30225.

6. Zhao M, Jiang Z, Cai H, Li Y, Mo Q, Deng L, Zhong H, Liu T, Zhang H, Kang JX, Feng F. 2020. Modulation of the Gut Microbiota during High-Dose Glycerol Monolaurate-Mediated Amelioration of Obesity in Mice Fed a High-Fat Diet. mBio 11.

7. Fortuoso BF, Galli GM, Griss LG, Armanini EH, Silva AD, Fracasso M, Mostardeiro V, Morsch VM, Lopes LQS, Santos RCV, Gris A, Mendes RE, Boiago MM, Da Silva AS. 2020. Effects of glycerol monolaurate on growth and physiology of chicks consuming diet containing fumonisin. Microb Pathog 147:104261.

8. Welch JL, Xiang J, Okeoma CM, Schlievert PM, Stapleton JT. 2020. Glycerol Monolaurate, an Analogue to a Factor Secreted by , Is Virucidal against Enveloped Viruses, Including HIV-1. mBio 11.

491 9. Fortuoso BF, Dos Reis JH, Gebert RR, Barreta M, Griss LG, Casagrande RA, de Cristo TG,
492 Santiani F, Campigotto G, Rampazzo L, Stefani LM, Boiago MM, Lopes LQ, Santos RCV,
493 Baldissera MD, Zanette RA, Tomasi T, Da Silva AS. 2019. Glycerol monolaurate in the diet of
494 broiler chickens replacing conventional antimicrobials: Impact on health, performance and
495 meat quality. Microb Pathog 129:161-167.
496 10. Pan D, Yu Z. 2014. Intestinal microbiome of poultry and its interaction with host and diet. Gut
497 microbes 5:108-119.
498 11. Jackman JA, Hakobyan A, Zakaryan H, Elrod CC. 2020. Inhibition of African swine fever
499 virus in liquid and feed by medium-chain fatty acids and glycerol monolaurate. Journal of
500 animal science and biotechnology 11:114.
501 12. Baltic B, Starčević M, Djordjevic J, Mrdović B, Markovic R. 2017. Importance of medium
502 chain fatty acids in animal nutrition. IOP Conference Series: Earth and Environmental Science
503 85:012048.
504 13. Jackman JA, Boyd RD, Elrod CC. 2020. Medium-chain fatty acids and monoglycerides as
505 feed additives for pig production: towards gut health improvement and feed pathogen
506 mitigation. Journal of animal science and biotechnology 11:44.
507 14. Abdollahi MR, Zaefarian F, Ravindran V. 2018. Feed intake response of broilers: Impact of
508 feed processing. Animal Feed Science and Technology 237:154-165.
509 15. Mustafa N. 2018. Biochemical Trails Associated with Different Doses of Alpha- Monolaurin
510 in Chicks. Advances in Animal and Veterinary Sciences 7.
511 16. Pié S, Lallès JP, Blazy F, Laffitte J, Sève B, Oswald IP. 2004. Weaning is associated with an
512 upregulation of expression of inflammatory cytokines in the intestine of piglets. The Journal

513 of nutrition 134:641-647.

514 17. Liu T, Li C, Zhong H, Feng F. 2021. Dietary medium-chain α-monoglycerides increase BW,
515 feed intake, and carcass yield in broilers with muscle composition alteration. Poultry science
516 100:186-195.

517 18. Liu T, Tang J, Feng F. 2020. Glycerol monolaurate improves performance, intestinal
518 development, and muscle amino acids in yellow-feathered broilers via manipulating gut
519 microbiota. Appl Microbiol Biotechnol 104:10279-10291.

520 19. Hong SM, Hwang JH, Kim IH. 2012. Effect of Medium-chain Triglyceride (MCT) on Growth
521 Performance, Nutrient Digestibility, Blood Characteristics in Weanling Pigs.
522 Asian-Australasian journal of animal sciences 25:1003-1008.

523 20. Masmeijer C, Rogge T, van Leenen K, De Cremer L, Deprez P, Cox E, Devriendt B, Pardon B.
524 2020. Effects of glycerol-esters of saturated short- and medium chain fatty acids on immune,
525 health and growth variables in veal calves. Preventive veterinary medicine 178:104983.

526 21. Peterson ML, Schlievert PM. 2006. Glycerol monolaurate inhibits the effects of Gram-positive
527 select agents on eukaryotic cells. Biochemistry 45:2387-2397.

528 22. Mo Q, Fu A, Deng L, Zhao M, Li Y, Zhang H, Feng F. 2019. High-dose Glycerol Monolaurate
529 Up-Regulated Beneficial Indigenous Microbiota without Inducing Metabolic Dysfunction and
530 Systemic Inflammation: New Insights into Its Antimicrobial Potential. Nutrients 11.

531 23. Nishimura Y, Moriyama M, Kawabe K, Satoh H, Takano K, Azuma Y-T, Nakamura Y. 2018.
532 Lauric Acid Alleviates Neuroinflammatory Responses by Activated Microglia: Involvement of
533 the GPR40-Dependent Pathway. Neurochemical research 43:1723-1735.

534 24. Zhang MS, Tran PM, Wolff AJ, Tremblay MM, Fosdick MG, Houtman JCD. 2018. Glycerol

monolaurate induces filopodia formation by disrupting the association between LAT and SLP-76 microclusters. Science signaling 11.

25. Sivinski SE, Mamedova LK, Rusk RA, Elrod CC, Swartz TH, McGill JM, Bradford BJ. 2020. Development of an macrophage screening system on the immunomodulating effects of feed components. Journal of animal science and biotechnology 11:89.

26. Sun S-C. 2017. The non-canonical NF-κB pathway in immunity and inflammation. Nature reviews Immunology 17:545-558.

27. Lee JY, Zhao L, Youn HS, Weatherill AR, Tapping R, Feng L, Lee WH, Fitzgerald KA, Hwang DH. 2004. Saturated fatty acid activates but polyunsaturated fatty acid inhibits Toll-like receptor 2 dimerized with Toll-like receptor 6 or 1. The Journal of biological chemistry 279:16971-16979.

28. Jiang J, Qi L, Lv Z, Jin S, Wei X, Shi F. 2019. Dietary Stevioside Supplementation Alleviates Lipopolysaccharide-Induced Intestinal Mucosal Damage through Anti-Inflammatory and Antioxidant Effects in Broiler Chickens. Antioxidants (Basel, Switzerland) 8.

29. Liu M, Chen X, Zhao H, Feng F. 2018. Effect of Dietary Supplementation with Glycerol Monolaurate on Growth Performance, Digestive Ability and Chicken Nutritional Components of Broilers. Food Science.

30. Shang Y, Regassa A, Kim JH, Kim WK. 2015. The effect of dietary fructooligosaccharide supplementation on growth performance, intestinal morphology, and immune responses in broiler chickens challenged with Salmonella Enteritidis lipopolysaccharides. Poultry science 94:2887-2897.

31. Cromarty R, Archary D. 2020. Inflammation, HIV, and Immune Quiescence: Leveraging on

Immunomodulatory Products to Reduce HIV Susceptibility. AIDS research and treatment 2020:8672850.

32. S L, Chaudhary S, R S R. 2017. Hydroalcoholic extract of Stevia rebaudiana bert. leaves and stevioside ameliorates lipopolysaccharide induced acute liver injury in rats. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie 95:1040-1050.

33. Valentini J, Da Silva AS, Fortuoso BF, Reis JH, Gebert RR, Griss LG, Boiago MM, Lopes LQS, Santos RCV, Wagner R, Tavernari FC. 2020. Chemical composition, lipid peroxidation, and fatty acid profile in meat of broilers fed with glycerol monolaurate additive. Food chemistry 330:127187.

34. Lugrin J, Rosenblatt-Velin N, Parapanov R, Liaudet L. 2014. The role of oxidative stress during inflammatory processes. Biol Chem 395:203-230.

35. Lucas K, Maes M. 2013. Role of the Toll Like receptor (TLR) radical cycle in chronic inflammation: possible treatments targeting the TLR4 pathway. Mol Neurobiol 48:190-204.

36. Wang W, Li Z, Han Q, Guo Y, Zhang B, D'Inca R. 2016. Dietary live yeast and mannan-oligosaccharide supplementation attenuate intestinal inflammation and barrier dysfunction induced by Escherichia coli in broilers. The British journal of nutrition 116:1878-1888.

37. Ott SJ, Musfeldt M, Wenderoth DF, Hampe J, Brant O, Fölsch UR, Timmis KN, Schreiber S. 2004. Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease. Gut 53:685-693.

38. Olejniczak-Staruch I, Ciążyńska M, Sobolewska-Sztychny D, Narbutt J, Skibińska M, Lesiak A. 2021. Alterations of the Skin and Gut Microbiome in Psoriasis and Psoriatic Arthritis.

579 International journal of molecular sciences 22.

580 39. Islam MR, Lepp D, Godfrey DV, Orban S, Ross K, Delaquis P, Diarra MS. 2019. Effects of
581 wild blueberry (Vaccinium angustifolium) pomace feeding on gut microbiota and blood
582 metabolites in free-range pastured broiler chickens. Poultry science 98:3739-3755.

583 40. Yu M, Mu C, Zhang C, Yang Y, Su Y, Zhu W. 2018. Marked Response in Microbial
584 Community and Metabolism in the Ileum and Cecum of Suckling Piglets After Early
585 Antibiotics Exposure. Frontiers in microbiology 9:1166.

586 41. Shi S, Qi Z, Jiang W, Quan S, Sheng T, Tu J, Shao Y, Qi K. 2020. Effects of probiotics on
587 cecal microbiome profile altered by duck Escherichia coli 17 infection in Cherry Valley ducks.
588 Microb Pathog 138:103849.

589 42. Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, deRoos P, Liu H, Cross JR, Pfeffer
590 K, Coffer PJ, Rudensky AY. 2013. Metabolites produced by commensal bacteria promote
591 peripheral regulatory T-cell generation. Nature 504:451-455.

592 43. Zhou Y, Wang Y, Quan M, Zhao H, Jia J. 2021. Gut Microbiota Changes and Their Correlation
593 with Cognitive and Neuropsychiatric Symptoms in Alzheimer's Disease. Journal of
594 Alzheimer's disease : JAD doi:10.3233/JAD-201497.

595 44. Jiang Z, Zhao M, Zhang H, Li Y, Liu M, Feng F. 2018. Antimicrobial Emulsifier-Glycerol
596 Monolaurate Induces Metabolic Syndrome, Gut Microbiota Dysbiosis, and Systemic
597 Low-Grade Inflammation in Low-Fat Diet Fed Mice. Molecular nutrition & food research 62.

598 45. Amer SA, A AN, Al-Khalaifah HS, AlSadek DMM, Abdel Fattah DM, Roushdy EM, Sherief
599 W, Farag MFM, Altohamy DE, Abdel-Wareth AAA, Metwally AE. 2020. Effect of Dietary
600 Medium-Chain α-Monoglycerides on the Growth Performance, Intestinal Histomorphology,

Amino Acid Digestibility, and Broiler Chickens' Blood Biochemical Parameters. Animals (Basel) 11.

46. Ren C, Wang Y, Lin X, Song H, Zhou Q, Xu W, Shi K, Chen J, Song J, Chen F, Zhang S, Guan W. 2020. A Combination of Formic Acid and Monolaurin Attenuates Enterotoxigenic Escherichia coli Induced Intestinal Inflammation in Piglets by Inhibiting the NF-κB/MAPK Pathways with Modulation of Gut Microbiota. J Agric Food Chem 68:4155-4165.

607 **TABLE 1** Ingredient composition and nutritional components of the basal diet

Items	Content (%)
Ingredient	
Corn	60
Soybean meal (43)	28.8
Corn protein flour (60)	5.3
Salt	0.16
Baking soda	0.2
Limestone	1.3
Dicalcium phosphate	0.75
Soybean oil	2.2
Vitamin premix	0.03
Mineral premix	0.2
Choline chloride (50%)	0.1
Methionine	0.23
Lysine (70%)	0.58
Threonine (98.5%)	0.134
Phytase (20000U)	0.02
Total	100
Nutritional level	
Metabolizable energy	2850 (kcal/kg)
Crude protein	21.5
Lysine	1.26
Methionine	0.57
Calcium	0.60
Total phosphorus	0.60
Available phosphorus	0.40

608 Provided for per kilogram of compound diet: vitamin A, 12000 IU; vitamin D3,
609 5000 IU; vitamin E, 80 mg; VK, 3.2 mg; vitamin B1, 3.2 mg; vitamin B2, 8.6 mg;
610 nicotinic acid, 65 mg; pantothenic acid, 20 mg; vitamin B6, 4.3 mg; biotin, 0.22 mg;
611 folic acid, 2.2 mg; vitamin B12, 0.017 mg; I, 1.25 mg; Fe, 20 mg; Mn, 120 mg; Se,
612 0.3 mg ; Zn, 110 mg. Nutrition level was the calculated value.

613 **TABLE 2** Nucleotide sequences for real-time PCR primers

Gene	Accession Number	Primer sequence, 5′→ 3′	Product size (bp)
IL-1β	NM_204524.1	GGTCAACATCGCCACCTACA CATACGAGATGCAAACCAGCAA	86
IL-6	NM_204628.1	CCTTTCAGACCTACCTGGAATT ACTTCATCGGGATTTATCACCA	130
TNF-α	NM_204267.1	TGTGTATGTGCAGCAACCCGTAGT GGCATTGCAATTTGGACAGAAGT	229
IFNγ	NM_205149.1	TGAGCCAGATTGTTTCGATG TCCTTTTGAAACTCGGAGGA	246
NF-κB	XM_015285418.2	CACGGAGGCTTGATCCTGTT CCGCTGTCCTGTCCATTCTT	96
TLR4	NM_001030693.1	AGTCTGAAATTGCTGAGCTCAAAT GCGACGTTAAGCCATGGAAG	190
ZO-1	XM_015278981.2	CTTCAGGTGTTTCTCTTCCTCCTCTC CTGTGGTTTCATGGCTGGATC	131
occludin	NM_205128.1	GCTCTGCCTCATCTGCTTCTT CCCATCCGCCACGTTCTTC	142
claudin-1	NM_001013611.2	CTGATTGCTTCCAACCAG CAGGTCAAACAGAGGTACAAG	140
claudin-2	NM_001277622.1	CCTGCTCACCCTCATTGGAG GCTGAACTCACTCTTGGGCT	145
GAPDH	NM_204305.1	GCCCAGAACATCATCCCA CGGCAGGTCAGGTCAACA	137
β-actin	NM_205518.1	CACCACAGCCGAGAGAGAAA CACAGGACTCCATACCCAAGAA	215
TBP	AF221563	AGCTCTGGGATAGTGCCACAG ATAATAACAGCAGCAAAACGCTTG	134

614 IL-1β, interleukin 1β, IL-6, interleukin 6; TNF-α, tumor necrosis factor α, TLR4,
615 toll-like receptor; NF-κB, nuclear factor kappa-B; IFN-γ, interferon γ; GAPDH,
616 glyceraldehyde-3-phosphate dehydrogenase; TBP, TATA-binding protein.

617 **TABLE 3** Effects of dietary treatment on growth performance in 7- and 14-day-old
618 broilers

Items	Treatments					*P*-value
	Control	300 mg/kg	600 mg/kg	900 mg/kg	1200 mg/kg	
BW (g/bird)						
1d	45.71±0.27	45.65±0.15	45.68±0.08	45.67±0.19	45.67±0.26	0.992
7d	154.82±6.25	151.19±6.66	148.71±4.84	150±8.19	152.99±2.22	0.437
14d	401.42±8.99	389.23±8.77	392.67±16.09	392.54±7.82	397.07±16.78	0.149
FI (g/bird)						
1 to 7d	145.74±7.85	148.6±2.78	143.01±6.03	150.34±1.05	155.88±5.04	0.123
7 to 14d	354.74±13.39^{ab}	337.05±18.91^{b}	367.62±4.35^{a}	375.46±13.88^{a}	366.22±27.4^{a}	0.013
1 to 14d	500.04±13.3^{bc}	485.75±17.41^{c}	511.82±17.56^{ab}	525.64±6.91^{a}	521.54±14.46^{a}	0.001
BWG (g/bird)						
1 to 7d	116.11±6.15	120.54±6.75	118.05±4.87	119.33±8.06	122.32±2.34	0.44
7 to 14d	237.6±9.36	221.38±8.04	228.95±12.93	227.54±4.09	229.08±15.14	0.202
1 to 14d	356.71±9.07	343.6±8.91	347±16.11	346.88±7.66	351.4±16.93	0.155
FCR (g/g)						
1 to 7d	1.25±0.04	1.24±0.06	1.21±0.02	1.26±0.1	1.27±0.04	0.343
7 to 14d	1.49±0.07	1.56±0.1	1.62±0.03	1.62±0.09	1.56±0.12	0.18
1 to 14d	1.41±0.04	1.41±0.07	1.47±0.03	1.50±0.01	1.48±0.07	0.13

619 BW, body weight; FI, feed intake; FCR, feed conversion ratio.
620 Values were expressed as mean ± SD (n = 6).
621 ^{a,b,c} Different superscripts in the same line indicate significant differences ($P < 0.05$).

622 **TABLE 4** Effects of dietary treatment on the jejunal morphology of 7- and 14-day-old
623 broilers

Items	Treatments					*P*-value
	CON	300 mg/kg	600 mg/kg	900 mg/kg	1200 mg/kg	
7 d of age						
VH (μm)	659.86±96.54	708.71±64.12	706.49±84.1	684.76±77.11	675.15±76.88	0.824
CD (μm)	107.01±17.21^{a}	101.01±15.22^{ab}	83.39±16.79^{bc}	67.32±11.39^{cd}	60.48±9.82^{d}	<0.001
VCR	6.24±1.02^{c}	7.11±0.95^{bc}	8.66±1.45^{b}	10.34±1.62^{a}	11.3±1.51^{a}	<0.001
14 d of age						
VH (μm)	745.08±71.15	697.3±86.11	670.03±99.88	686.95±28.38	708.35±61.9	0.765
CD (μm)	102.33±12.34^{a}	89.9±7.99^{b}	73.28±0.86^{c}	66.33±4.16^{c}	69.1±6.76^{c}	<0.001
VCR	7.3±0.23^{b}	7.84±1.39^{b}	8.88±1.58^{ab}	10.37±0.47^{a}	10.34±1.44^{a}	0.011

624 VH, villus height; CD, crypt depth; VCR, villus height to crypt depth ratio.
625 Values were expressed as mean ± SD (n = 6).
626 ^{a,b,c,d} Different superscripts in the same line indicate significant differences ($P < 0.05$).

627 **TABLE 5** Effects of dietary treatment on biochemical indicators in the sera and jejuna
628 of 7- and 14-day-old broilers

Items	Treatments					*P*-value
	CON	300mg/kg	600mg/kg	900mg/kg	1200mg/kg	
7 d of age (ng/mL)						
IL-1β	597.46±33.97^{a}	559.36±45.06^{ab}	531.91±34.96^{b}	541.96±37.13^{b}	525.78±52.65^{b}	0.028
IL-6	29.05±2.08	28.9±1.71	28.62±1.67	29.73±1.1	29.57±1.05	0.897
TNF-α	81.36±6.26^{a}	79.03±3.78^{ab}	78.27±2.9^{ab}	75.44±7.37^{bc}	72.75±3.49^{c}	0.029
IgG	1934.38±153.59	1911.25±107	1832.5±175.64	1953.13±157.39	1829.38±160.03	0.35
sIgA	26.01±3.4	26.48±3.09	25.7±2.6	26.6±2.61	25.32±2.82	0.891
Muc2	213.65±8.99	209.97±8.15	201.65±13.8	202.8±17.57	197.98±13.87	0.219
14 d of age (ng/mL)						
IL-1β	629.71±27.08^{a}	583.17±35.5^{ab}	566.84±39.61^{b}	543.68±56.89^{b}	560.59±51.03^{b}	0.018
IL-6	29.49±2.86	29.63±0.68	29.63±1.22	30.96±1.94	30.2±1.23	0.746
TNF-α	76.9±8.37	73.69±3.46	78.36±1.64	77.96±2.12	78.21±3.92	0.162
IgG	1898.13±195.47^{b}	1813.13±145.67^{b}	1941.88±167.71^{ab}	1939.38±202.95^{ab}	2115±212.94^{a}	0.042
sIgA	24.56±1.85	25.1±2.53	26.91±1.38	25.81±2.13	25.73±1.93	0.268
Muc 2	196.06±8.96^{b}	192.53±12.58^{b}	202.68±15.33^{ab}	201.31±12.18^{ab}	212.2±10.18^{a}	0.028

629 IL-1β, interleukin 1β; TNF-α, tumor necrosis factor α; IgG, immunoglobulin G; sIgA,
630 secreted immunoglobulin A; Muc 2, mucin 2.
631 Values were expressed as mean ± SD (n = 12).
632 ^{a,b,c} Different superscripts in the same line indicate significant differences ($P < 0.05$).

633 **TABLE 6** Effects of dietary treatment on antioxidant capacity in 7- and 14-day-old
634 broilers

Items	Treatments					*P*-value
	CON	300 mg/kg	600 mg/kg	900 mg/kg	1200 mg/kg	
Serum, 7 d of age						
MDA (nmol/ml)	5.88±1.87	5.29±0.68	5.99±1.36	5.67±0.43	5.08±0.68	0.303
SOD (U/ml)	67.41±14.18^{b}	77.19±15.5^{ab}	65.4±4.71^{b}	82.87±11.1^{ab}	93.84±17.69^{a}	0.039
T-AOC (mmol/ml)	0.56±0.06	0.54±0.11	0.52±0.08	0.58±0.1	0.63±0.11	0.269
14 d of age						
MDA (nmol/ml)	6.6±1.39^{a}	4.64±1.23^{b}	4.07±0.72^{b}	4.51±0.56^{b}	4.95±1.12^{b}	0.005
SOD (U/ml)	72.51±6.54	84.33±28.02	89.43±9.3	101.29±26.88	111±21.96	0.071
T-AOC (mmol/ml)	0.41±0.05^{b}	0.47±0.05^{ab}	0.49±0.11^{ab}	0.52±0.04^{a}	0.5±0.04^{a}	0.005
Jejunum, 7 d of age						
MDA (nmol/mgprot)	0.36±0.17	0.65±0.36	0.84±0.67	0.69±0.47	0.65±0.28	0.170
SOD (U/mgprot)	2.47±0.48^{d}	3.35±0.55^{c}	4.11±0.95^{ab}	3.6±0.59^{bc}	4.49±0.84^{a}	<0.001
T-AOC (mmol/mgprot)	0.08±0.01^{b}	0.1±0.02^{b}	0.12±0.03^{a}	0.1±0.02^{b}	0.16±0.08^{a}	0.029
14 d of age						
MDA (nmol/mgprot)	0.98±0.21^{a}	0.6±0.08^{b}	0.56±0.16^{b}	0.55±0.16^{b}	0.6±0.14^{b}	<0.001
SOD (U/mgprot)	3.72±0.65	3.77±0.7	3.76±0.9	4.01±0.74	3.39±0.55	0.556
T-AOC (mmol/mgprot)	0.08±0.01	0.08±0.01	0.08±0.02	0.08±0.02	0.07±0.01	0.686

635 MDA, malondialdehyde; SOD, superoxide dismutase; T-AOC, total antioxidant
636 capacity; mgprot, mg of protein.
637 Values were expressed as mean ± SD (n = 12).
638 ^{a,b,c,d} Different superscripts in the same line indicate significant differences ($P < 0.05$).

639 **TABLE 7** Effects of dietary treatment on jejunal gene expression of 7- and
640 14-day-old broilers

Item	Treatment					*P*-value
	CON	300 mg/kg	600 mg/kg	900 mg/kg	1200 mg/kg	
7 d of age						
IL-1β	1±0.19^{a}	0.77±0.23^{ab}	0.96±0.25^{a}	0.8±0.34^{ab}	0.55±0.07^{b}	0.019
IL-6	1±0.24	1.49±0.23	1.55±0.35	1.32±0.16	1.16±0.27	0.281
TNF-α	1±0.29	1.01±0.23	0.92±0.27	0.76±0.06	0.72±0.05	0.256
IFN-γ	1±0.31^{a}	0.96±0.57^{ab}	1.28±0.33^{a}	0.85±0.32^{ab}	0.59±0.19^{b}	0.047
ZO-1	1±0.1^{b}	1.13±0.19^{ab}	1.31±0.17^{a}	1.17±0.21^{ab}	1.03±0.07^{ab}	0.029
occludin	1±0.18^{b}	1.54±0.12^{a}	1.31±0.09^{a}	1.04±0.11^{b}	1.31±0.32^{a}	0.002
claudin-1	1±0.13	0.99±0.18	1.29±0.34	1.02±0.35	1.35±0.66	0.374
claudin-2	1±0.29	0.96±0.32	1.22±0.48	1.22±0.43	1.17±0.28	0.689
TLR4	1±0.2	1.06±0.32	0.91±0.07	0.83±0.21	1.01±0.13	0.501
NF-κB	1±0.13	0.97±0.25	1.11±0.22	1.01±0.23	0.88±0.17	0.443
14 d of age						
IL-1β	1±0.55	0.62±0.18	0.75±0.29	0.79±0.27	0.93±0.21	0.208
IL-6	1±0.18^{a}	0.87±0.26^{ab}	0.84±0.17^{ab}	1.09±0.23^{a}	0.65±0.23^{b}	0.037
TNF-α	1±0.2	0.94±0.21	1.04±0.15	0.88±0.26	1.07±0.24	0.548
IFN-γ	1±0.51^{a}	0.61±0.19^{ab}	0.52±0.13^{b}	0.51±0.25^{b}	0.45±0.23^{b}	0.019
ZO-1	1±0.08	1.02±0.16	1.13±0.18	1.2±0.25	1.34±0.35	0.111
occludin	1±0.12^{abc}	0.86±0.19^{bc}	0.77±0.16^{c}	1.26±0.13^{a}	1.13±0.2^{ab}	0.001
claudin-1	1±0.37	0.92±0.25	0.88±0.18	1.09±0.36	0.84±0.21	0.494
claudin-2	1±0.16	0.99±0.15	1±0.26	1.24±0.39	1.24±0.44	0.419
TLR4	1±0.35^{a}	0.69±0.18^{ab}	0.63±0.15^{b}	0.82±0.14^{ab}	0.65±0.09^{b}	0.049
NF-κB	1±0.33	0.7±0.12	0.79±0.08	0.84±0.13	0.8±0.17	0.093

641 IL-1β, interleukin 1β, IL-6, interleukin 6; TNF-α, tumor necrosis factor α, TLR4,
642 toll-like receptor; NF-κB, nuclear factor kappa-B; IFN-γ, interferon γ; GAPDH,
643 glyceraldehyde-3-phosphate dehydrogenase; TBP, TATA-binding protein.
644 Values were expressed as mean ± SD (n = 6).
645 ^{a,b,c} Different superscripts in the same line indicate significant differences ($P < 0.05$).

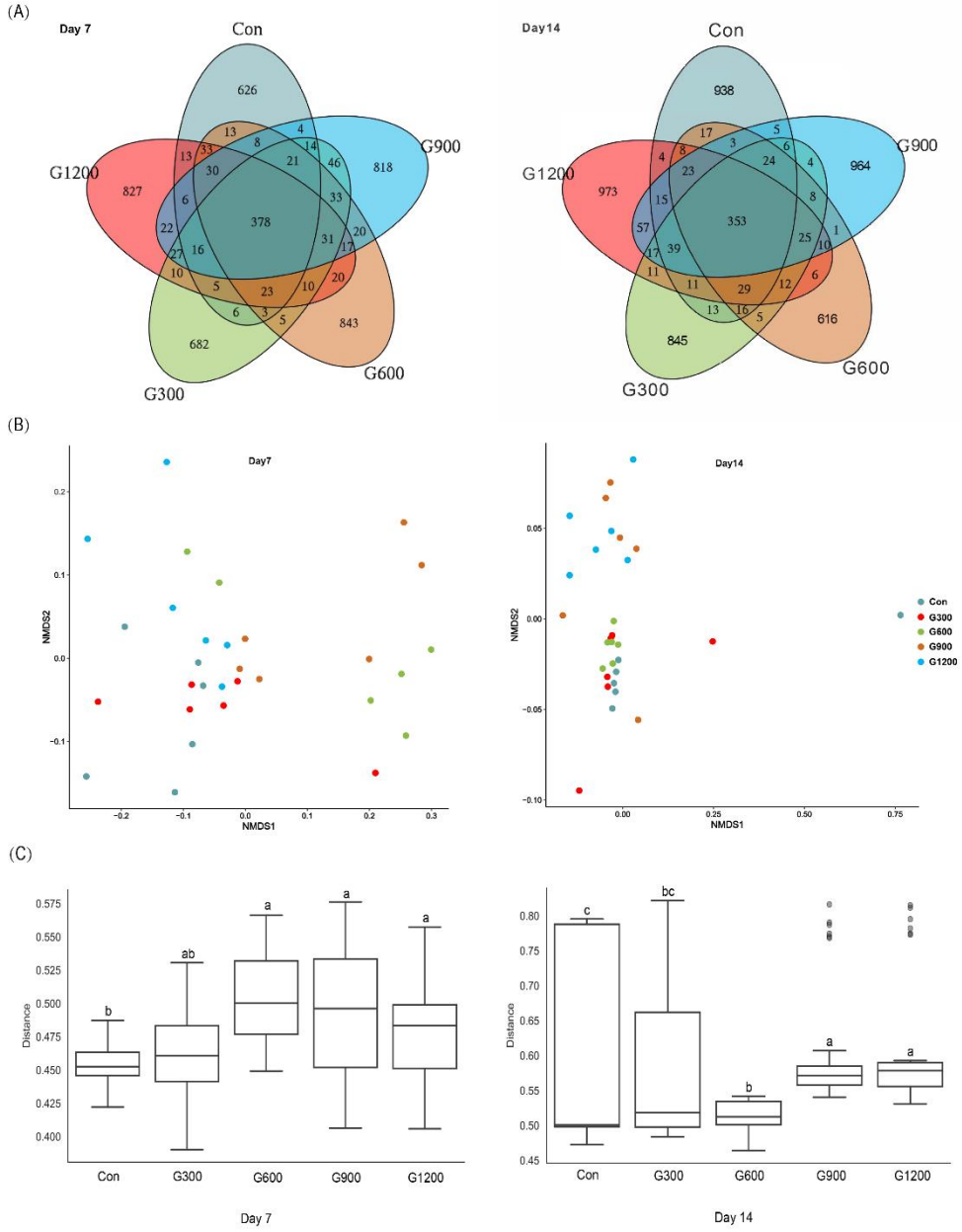
646 **TABLE 8** Effects of GML on cecal microbiota α-diversity of broilers

Item	Treatment					*P*-value
	CON	300mg/kg	600mg/kg	900mg/kg	1200mg/kg	
7 d of age						
Chao 1	410.11±33.93^{b}	435.31±78.4^{ab}	497.67±53.9^{a}	506.31±71.22^{a}	501.08±61.47^{a}	0.029
Pd	28.09±2.25^{ab}	27.39±4.35^{ab}	26.76±3.77^{b}	27.28±1.88^{b}	31.02±2.54^{a}	0.112
Observed	409.67±34^{b}	434.17±78.23^{ab}	496.33±54.08^{a}	505±70.25^{a}	499.5±60.82^{a}	0.029
Shannon	6.22±0.31	6.29±0.62	6.67±0.6	6.7±0.59	6.6±0.42	0.393
Simpson	0.96±0.01	0.96±0.03	0.97±0.02	0.97±0.02	0.97±0.01	0.649
14 d of age						
Chao 1	548.04±45.24^{a}	550.24±83.38^{a}	452.16±48.86^{b}	548.93±109.31^{ab}	612.15±45.41^{a}	0.018
Pd	34.35±13.06^{ab}	32.23±8.92^{ab}	28.18±2.59^{b}	32.16±4.49^{ab}	35.03±4.08^{a}	0.111
Observed	547.33±45.56^{a}	548.67±83.56^{a}	451.33±48.63^{b}	547.33±109.79^{ab}	611±45.22^{a}	0.018
Shannon	5.36±0.57^{a}	5.03±1.01^{ab}	4.51±0.36^{b}	4.99±0.51^{ab}	5.57±0.46^{a}	0.033
Simpson	0.89±0.06^{ab}	0.83±0.13^{ab}	0.85±0.04^{b}	0.87±0.02^{b}	0.91±0.03^{a}	0.153

647 Values were expressed as mean ± SD (n = 6).

648 ^{a,b} Different superscripts in the same line indicate significant differences according to

649 the Kruskal-Wallis test ($P < 0.05$).



650

651 **FIG 1** Dietary GML altered the composition and community diversity of cecal
652 microbiota. (A) Venn diagram between treatments on OTUs level. (B) NMDS
653 analysis based on unweighted Unifrac distance. (C) PERMANOVA analysis based on
654 unweighted Unifrac distance. Con, basal diet; G300, 600, 900, and 1200, basal diets
655 complemented with 300, 600, 900, or 1200 mg/kg GML.

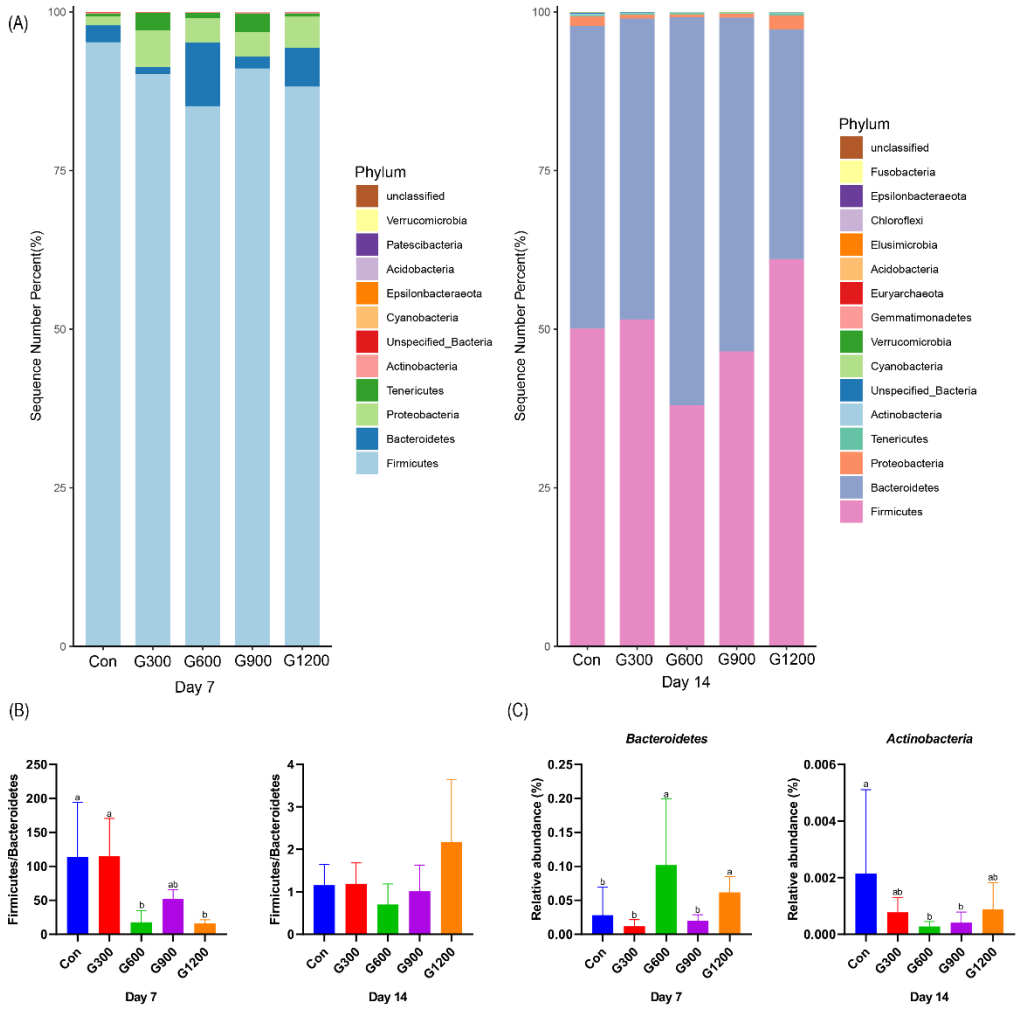


FIG 2 Alteration of cecal microbiota on the phylum level. (A) The top 20 phyla in the relative abundance of each group. (B) The ratio of Firmicutes/Bacteroidetes. (C) Relative abundance of *Bacteroidetes* and *Actinobacteria*. Different superscripts indicate significant differences according to the Kruskal-Wallis test ($P < 0.05$). Con, basal diet; G300, 600, 900, and 1200, basal diets complemented with 300, 600, 900, or 1200 mg/kg GML.

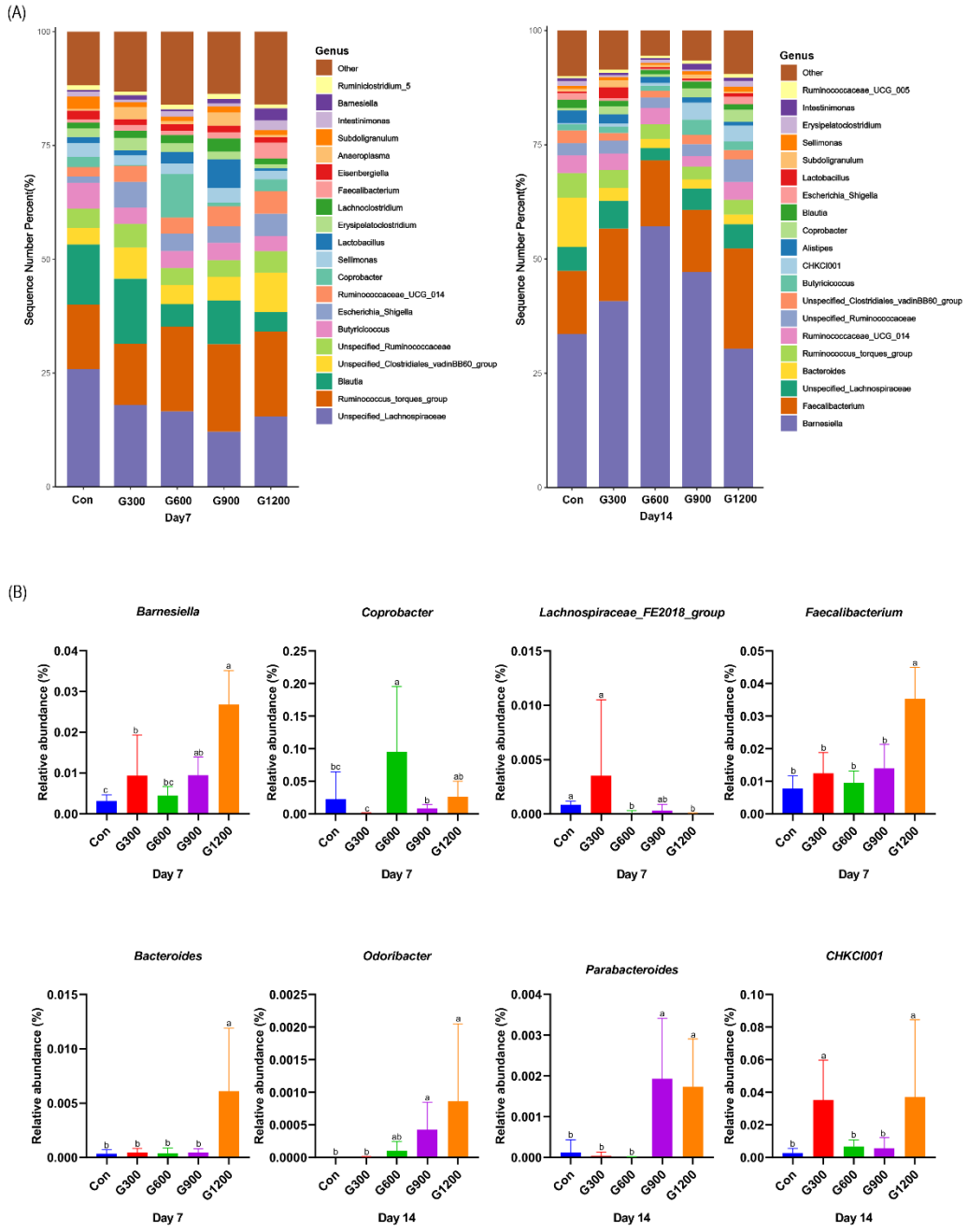


FIG 3 Alteration of cecal microbiota on the genus level. (A) Relative abundance of top 20 bacterial genera presents in each group. (B) The relative abundance differs significantly among groups at the genus level. Different superscripts indicate significant differences according to the Kruskal-Wallis test ($P < 0.05$). Con, basal diet; G300, 600, 900, and 1200, basal diets complemented with 300, 600, 900, or 1200 mg/kg GML.

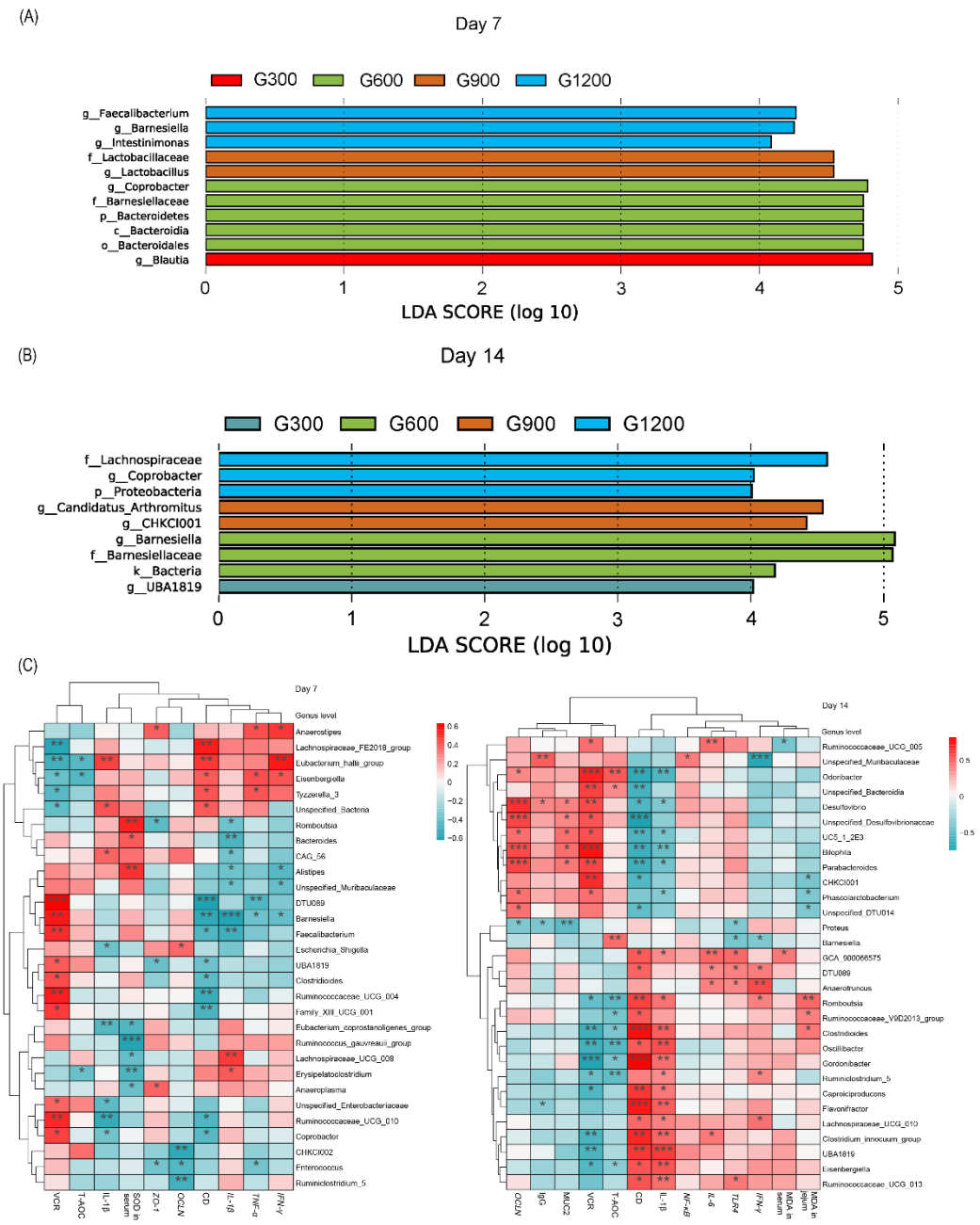


FIG 4 (A) LEfSe analysis of cecal microbiota (LDA score is greater than 4). (B) Heatmap of correlation among cecal microbiota, inflammatory factors, tight junctions, antioxidant enzymes, and intestinal integrity by Spearman analysis. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. Con, basal diet; G300, 600, 900, and 1200, basal diets complemented with 300, 600, 900, or 1200 mg/kg GML.