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Effects of Dietary Glycerol Monobutyrate Supplementation on Egg Performance, Biochemical Indices, and Gut Microbiota of Aged Hens

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This experiment aimed to determine the effect of dietary supplementation with glycerol monobutyrate (**GMB**) on egg-laying performance, biochemical indicators, and gut microflora at the late stage of laying hens. A total of 252 healthy Dawu Golden Phoenix laying hens with no difference in body weight were selected and randomly divided into two groups: (1) control group (**CG**), corn-soybean meal diet, (2) 500 mg glycerol monobutyrate/kg added to the basal diet. Six replicates were set up for each treatment group, with 21 birds per replicate. The trial started at week 55 and lasted for 8 weeks. Compared to the control group, the supplementation with GMB increased egg weight ($P = 0.03$), shell thickness ($P = 0.03$) and decreased egg breaking rate ($P = 0.04$). There was no significant difference in egg production rate, feed-to-egg ratio, egg shape index, eggshell strength, and Haugh unit between the two groups. In addition, dietary GMB decreased the levels of aspartate aminotransferase ($P = 0.03$) and total bilirubin ($P = 0.02$) in serum, and increased total antioxidant capacity ($P = 0.03$) and total superoxide dismutase ($P = 0.02$). However, alpha diversity indices (Ace, Chao1, Shannon, Simpson, goods_coverage, and PD_whole tree) were not different between the two groups. Notably, dietary GMB significantly decreased the abundances of *Proteobacteria* at the phylum level and the abundances of *Enterobacter* at the genus level ($P < 0.01$), but there was no significant difference in the composition of other cecal microbiota. In summary, the present study revealed that supplementation with 500 mg/kg glycerol monobutyrate improved egg weight, eggshell quality, and antioxidant capacity in serum, but its effect on cecal microbiota composition was limited.

Keywords: glycerol monobutyrate, laying hens, laying performance, egg quality, biochemical indices, microbiota

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INTRODUCTION

The production performance and eggshell quality of laying hens in the late laying period (40–48 weeks old) decreased with age (Bain et al., 2016). In addition, poor health of older hens have also resulted in poor egg quality, thinner shells, increased breakage rates, reduced egg white height, and poor egg flavor (Gan et al., 2020). Short-chain fatty acids (SCFAs) are a feed additive capable of controlling poultry pathogens (Ricke, 2003). Butyric acid is a 4-carbon SCFA, its glyceride form is easy to handle because it does not have the unique unpleasant odor of free acids, and it also facilitates release into the digestive tract (Sampugna et al., 1967).

Recently published research has shown that supplementation of monobutyric acid in the diet increases egg weight and tends to reduce egg breakage in late-production Qingyuan chickens (Feng et al., 2021). Meantime, dietary glycerol monolaurate improves reproductive performance, feed efficiency, and egg quality in aged hens associated with gut microbiota alteration (Liu et al., 2020). The addition of glyceryl butyrate to the diet could improve slaughter weight and feed efficiency of broilers (Antongiovanni et al., 2007). However, there is no effect on the average daily gain and feed efficiency of broilers by dietary monobutyrate (Bedford et al., 2017). Surprisingly, the mixture of monobutyrin and tributyrin improved blood biochemical parameters in broilers, especially in lipid catabolism (Yin et al., 2016).

The healthy development of the gut is an important condition for the healthy production of the animal body. Chickens have a complex microbiota in the cecum that interacts closely with the host and ingested feed (Pan and Yu, 2014). Growing evidence also links gut microbiota and function to weight gain, feed utilization conversion, and chicken health (Angelakis, 2017). The dysbiosis of broiler chickens is caused by the prohibition of antibiotics in feed, dietary changes, and environmental stresses in modern broiler production. In the case of intestinal flora imbalance in the body, it is usually accompanied by an increase in the level of *Proteobacteria* (Pinacchio et al., 2018). *Proteobacteria* mainly consists of many gram-negative bacteria such as *Vibrio cholerae*, *Helicobacter pylori*, *Salmonella*, and *Escherichia coli*, which could result in diarrhea, gastritis, vomiting, gastrointestinal ulcers, and even death; posing a great threat to animal health (Li et al., 2021). Butyric acid has been reported to have antibacterial properties, it can diffuse through the bacterial cell membrane and dissociate within the bacterial cell, resulting in a drop in the intracellular pH of the bacterial cell and eventual death (Hanna, 2019). Derivatives of butyric acid have been added to broiler diets to replace antibiotics, with glycerol butyrate being typical. Glyceryl butyrate has been reported to maintain broiler performance when encountering coccidiosis (Leeson et al., 2005) and can reduce Salmonella Enteritidis infection and improve growth performance under stress (Zhang et al., 2011). Alpha monoglycerides of these SCFAs have been reported to have stronger antibacterial effects, and their addition to the diet may benefit chicken gut health and growth performance (Namkung et al., 2011). Many studies have been conducted on how butyrin and other forms of butyric acid affect broiler performance. However, studies on the effects of butyrin on egg quality, blood biochemical markers, and gut microbiota in laying hens are limited. This study aimed to investigate how glycerol monobutyrate affects egg quality, blood biochemical parameters, and cecal microbiota in late laying hens.

MATERIALS AND METHODS

Birds, Experimental Design, and Diets

A total of 252 (55-wk-old) Dawu Golden Phoenix laying hens were randomly divided into two groups (21 hens per replicate, 6 replicates per group) and fed with a basal diet or a basal diet containing 500 mg/kg of GMB. The experiment started at week 55 and lasted for 8 wk. The basal diet was formulated according to the Nutrient Requirements for laying hens (2012). During the study, the birds were housed in three-tiered cages with an overall shape like the letter A when viewed from the side and shared a room cleaned and disinfected daily, maintained at 26 ± 2°C and 60–65% humidity with a 16 h light regime. Feed (100 g per hen per day) was provided at 7:00 a.m. and 2:00 p.m. and eggs were collected at 5:00 p.m. The birds had free access to drink water. Feeding, egg collection, and weighing of eggs were conducted daily.

Productive Performance and Egg Quality

Feed intake, number of eggs, and egg breaking were recorded daily during 55–62 wk. The egg breaking rate was calculated based on the number of broken eggs. The egg production rate, feed-to-egg ratio, and average egg weight were calculated per week. In the last week of the experiment, 36 eggs from each group (6 eggs per replicate) were randomly selected. Haugh units (Egg Analyzer, Orka Food Technology Ltd., Israel), shell breaking strength (Egg Force Reader, Orka Food Technology Ltd.), and shell thickness (Eggshell Thickness Gauge, Orka Food Technology Ltd.) were determined from these 72 eggs. The egg width to length ratio was calculated as the egg shape index (%).

Serum Biochemical Indices and Antioxidant Parameters

One bird was randomly selected from each replicate and blood was collected after feed deprivation for 12 h at the end of the experiment (Feng et al., 2021). Blood samples were collected from the pterygoid vein and serum was separated after 2 h of quiescence at 4°C (Gong et al., 2021). Triglycerides, total cholesterol, total bilirubin, high-density lipoprotein, low-density lipoprotein, aspartate aminotransferase, and alanine aminotransferase in serum were detected by kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). Antioxidant parameters analyzed according to the instruction manual of the Nanjing Jiancheng Bioengineering Institute kit included malondialdehyde (MDA), total antioxidant capacity (T-AOC), total superoxide dismutase (T-SOD), and glutathione peroxidase (GSH-PX). After the blood collection, the selected chickens were sacrificed by cervical dislocation and bled. The intestinal contents of the left and right cecum were aseptically collected from each chicken in a capped vial and immediately snap frozen in liquid nitrogen. The samples were then stored at −80°C until further analysis.

Cecal Digesta DNA Extraction and 16S rRNA Sequencing Analysis

Cecal microbiota evaluation was carried on the selected six older laying hens per group at the end of the study. After being snap frozen in liquid nitrogen, the cecal contents were extracted aseptically and kept at −80°C. The cetyltrimethylammonium bromide method was used to extract total genome DNA from cecal digesta. 1.5% agarose gel electrophoresis has been used to determine DNA content and purity. The primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVG GGTATCTAATCC-3′) were used to amplify the V3/V4 region of the 16S rRNA gene. To obtain the high-quality clean reads, paired-end reads were merged using Fast Length Adjustment of Short Reads software (V1.2.7) and quality filtering on the raw sequences was conducted on a quality control pipeline using the Quantitative Insight into Microbial Ecology (QIIME) tool kit (Caporaso et al., 2010). At 97 percent similarity, high-quality sequences were grouped into operational taxa (OTUs), and representative OTU sequences were assigned taxonomy using the SILVA database (Quast et al., 2013). A Venn diagram had been used to visualize shared and unique OTUs among three groups. Package vegan was used to calculate the alpha diversity, which was then displayed utilizing GraphPad prism 9.0. A principal coordinate analysis (PCOA) based on Bray-Curtis distance was performed for beta-diversity analysis, with statistical significance evaluated using the R package's permutational multivariate analysis of variance (*t*-test). Linear discriminant analysis effect size and STAMP with *t*-test were being used to investigate differences in microbiota relative abundances (Parks et al., 2014).

Statistical Analysis

All data were expressed as means ± SD, using an independent samples *t*-test with SPSS 25.0 software (SPSS, Inc., Chicago, IL), including fixed effects treatments in the model, and presented using GraphPad Prism version 9 (GraphPad Software, La Jolla, CA). A $P < 0.05$ was considered significant ($P < 0.05$, $P < 0.01$), and $0.05 < P < 0.10$ was discussed as tendencies.

RESULTS

Effect of Dietary GMB on Productive Performance and Egg Quality

In this study, the egg weight and shell thickness of the GMB group were significantly higher than that of the control group (**Table 1**; $P < 0.05$). There was no significant difference between the two groups in egg shape index, eggshell strength, and Haugh unit. However, the egg breaking rate of the treatment group decreased by 0.16% compared with the control group (**Table 1**; $P < 0.05$). These results indicated that dietary GMB could improve egg weight and shell thickness and reduce egg breaking rate in the late laying period.

TABLE 1 | Effects of glycerol monobutyrat supplementation on productive performance and egg quality.

Items	CG	GMB	SEM	*P*-value
Productive performance				
Egg production rate, %	82.02 ± 5.28	85.59 ± 4.47	1.22	0.17
Egg weight, g	60.80 ± 0.24	61.07 ± 0.16	0.55	0.03
Feed-to-egg ratio, g/g	2.01 ± 0.14	1.92 ± 0.10	0.31	0.15
Egg breaking rate, %	0.65 ± 0.16	0.49 ± 0.13	0.04	0.04
Egg quality				
Egg shape index	1.31 ± 0.01	1.31 ± 0.01	0.04	0.95
Shell breaking strength, kg/cm^2	3.81 ± 0.08	3.82 ± 0.18	0.04	0.95
Shell thickness, mm	0.38 ± 0.02	0.41 ± 0.03	0.01	0.03
Haugh units	71.83 ± 7.34	76.82 ± 3.06	1.62	0.16

CG, control group; GMB, glycerol monobutyrat.

TABLE 2 | Effects of glycerol monobutyrat supplementation on serum biochemical indices and antioxidant parameters.

Items	CG	GMB	SEM	*P*-value
Serum biochemical indices				
Triglycerides, mmol/L	2.87 ± 0.64	3.44 ± 0.64	0.18	0.15
Total bilirubin, μmol/L	110.54 ± 14.53	90.72 ± 11.14	3.74	0.02
Total cholesterol, mmol/L	3.62 ± 1.06	4.06 ± 0.61	0.25	0.40
High density lipoprotein, mmol/L	5.18 ± 0.86	6.17 ± 1.36	0.33	0.16
Low density lipoprotein, mmol/L	0.32 ± 0.09	0.29 ± 0.06	0.02	0.45
Aspartate aminotransferase (AST), U/L	28.27 ± 6.68	18.74 ± 6.16	1.16	0.03
Alanine aminotransferase (ALT), U/L	12.85 ± 3.25	7.99 ± 4.45	1.12	0.06
Antioxidant parameters				
MDA, nmol/ml	5.65 ± 1.38	5.48 ± 1.30	0.39	0.84
T-AOC, U/ml	4.40 ± 1.68	6.73 ± 1.57	0.47	0.03
T-SOD, U/ml	75.04 ± 7.42	88.20 ± 8.15	2.25	0.02
GSH-PX, U/ml	594.36 ± 56.67	652.50 ± 61.44	17.06	0.12

CG, control group; GMB, glycerol monobutyrat; MDA, malondialdehyde; T-AOC, total antioxidant capacity; T-SOD, total superoxide dismutase; GSH-PX, glutathione peroxidase.

Effect of Dietary GMB on Serum Biochemical Indices and Antioxidant Parameters

The aspartate aminotransferase and total bilirubin of the treatment group were significantly lower than that of the control group (**Table 2**; $P < 0.05$). Compared with the control group, the alanine aminotransferase, in the GMB group tended to decrease (**Table 2**; $P = 0.056$). The total antioxidant capacity and total superoxide dismutase of the GMB group were significantly increased (**Table 2**; $P < 0.05$), suggesting that part of the antioxidant parameters of laying hens could be improved by GMB supplementation.

Effect of Dietary GMB on Microbial Composition of Cecal Contents

A total of 2,481 operational taxonomic units (OTUs) were received from the two groups, and 1,029 and 396 OTUs were, respectively, found only in the control group and GMB group

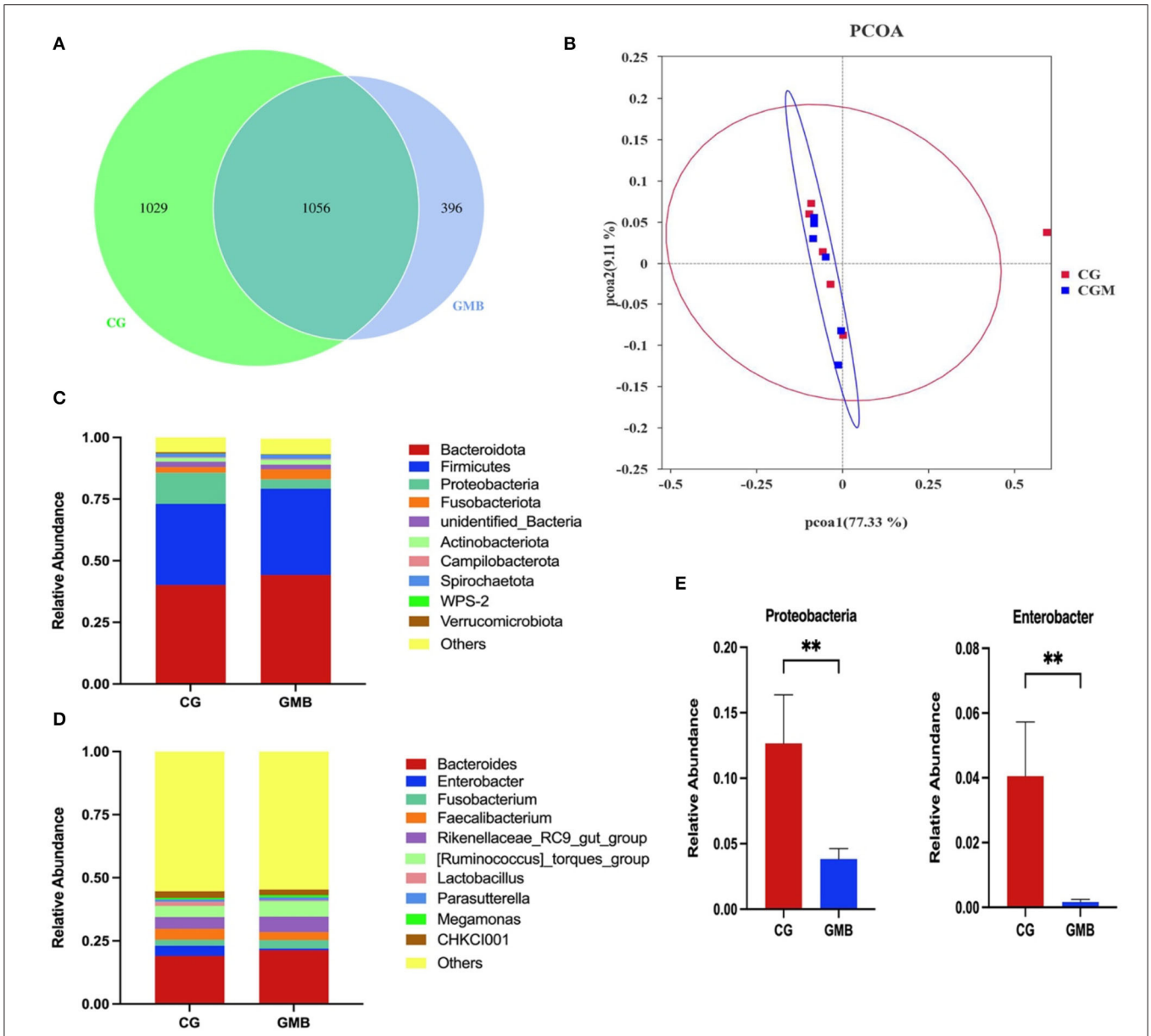

FIGURE 1 | Effects of dietary glycerol monobutyrat on the microbial composition of cecal contents. **(A)** Venn diagram of the number of operational taxonomic units (OTUs); **(B)** Principal coordinate analysis (PCOA) of the cecal microbiota; **(C)** Phylum-level taxonomic composition of the cecal microbiota; **(D)** Genus-level taxonomic composition of the cecal microbiota. **(E)** Relative abundance of *Proteobacteria* and *Enterobacter*. CG, control group; GMB, glycerol monobutyrat. Asterisks indicate significant differences (**$P < 0.01$).

(**Figure 1A**). Alpha diversity indices including Ace, Chao1, Shannon, Simpson, goods_coverage, and PD_whole tree were not affected by GMB supplementation (**Table 3**). The Bray-Curtis similarity method was used for PCOA analysis. The first principal component (PCOA1) and the second principal component (PCOA2) explained 77.33 and 9.11% of the microbial diversity variation, respectively (**Figure 1B**). The principal coordinate analysis diagram shows that the samples in the control group and GMB group were densely clustered and not far away from each other. The taxonomic analysis reflected that the structure of cecal flora did not change after GMB treatment. At the phylum level, *Bacteroidetes* (>40%) and *Firmicutes* (>32%) are the first 2 most predominant phylum (**Figure 1C**). Proteobacteria were significantly reduced in the GMB group (**Figure 1E**; $P < 0.01$), while other phyla did not change significantly. The core gut microbiota in both groups was similar at the genus level. At the genus level, *Bacteroidota* dominates (>35%), followed by *Firmicutes* (>32%), *Fusobacteriota* (>2%), and *unidentified_Bacteria* (>2%) and the rest genera listed were all below 2% (**Figure 1D**). The supplementation of glycerol monobutyrate did not affect microbiota composition at genus levels other than *Enterobacter* whereas the GMB

TABLE 3 | Effects of glycerol monobutyrat supplementation on alpha diversity indexes of cecal microbiota.

Items	CG	GMB	SEM	*P*-value
Ace	1012.25 ± 129.17	923.07 ± 65.11	29.53	0.16
Chao1	994.57 ± 115.81	910.15 ± 39.47	24.98	0.12
Shannon	6.84 ± 0.49	6.92 ± 0.29	0.12	0.74
Simpson	0.97 ± 0.03	0.98 ± 0.01	0.01	0.39
Goods_coverage	1.00 ± 0.00	1.00 ± 0.00	0.00	1.00
PD_whole tree	82.47 ± 53.67	57.08 ± 4.83	10.18	0.24

CG, control group; GMB, glycerol monobutyrat.

supplementation significantly reduced the relative abundance (**Figure 1E**; $P < 0.01$).

DISCUSSION

In the production process of laying hens, egg production, and egg quality are the most direct and important economic issues. Eggshell thickness and eggshell strength are very important for egg quality. Thicker eggshells have a great impact on the egg breaking rate, and also make the eggs have good pressure resistance, which is convenient for long-distance transportation and storage. Dawu Golden Phoenix laying hens will be culled after 72 weeks when the egg production rate is below 80% (Li, 2021). In the present study, our results showed that dietary supplementation with GMB did not significantly improve egg production and reduce the feed-to-egg ratio. The effects of glyceryl butyrate supplementation on broiler performance vary widely. Bedford et al. (2017) observed that the average daily gain and feed conversion ratio in broilers were not significantly difference by dietary monobutyric acid levels ranging from 500 ppm to 3,000 ppm. Feng et al. (2021) reported that the addition of 250 mg/kg of monobutyric acid did not affect performance and feed conversion ratio. However, some researchers have observed improved growth performance (Antongiovanni et al., 2007). Hu and Guo (2007) observed weight gain in broilers from 0 to 21 days. Nollet et al. (2002) found that 500 mg/kg of sodium butyrate supplementation did not affect average egg weight, but improved egg production and feed conversion. When assessing the effects of butyrate supplementation, different responses were attributable to supplementation levels, dietary composition, age, and health status (Cerisuelo et al., 2014). More researchers have observed that dietary butyrate is beneficial to eggshell quality and thus reduces egg breakage. Feng et al. (2021) observed that monobutyric acid could improve egg weight and reduce egg breaking rate, but had no significant effect on eggshell strength and eggshell thickness. Hanna (2019) observed an increased effect of butyrate (550 mg/kg) on eggshell strength. Butyrate (added at 185 mg/kg) can enhance eggshell strength and reduce the number of deformed eggs in older hens (Sengor et al., 2007). Hu and Guo (2007) noted butyric acid is a SCFA and has water-fat amphiphilicity and can be absorbed and utilized by intestinal epithelial cells in the intestine, thereby providing more energy for the body, which may be the reason for improving egg quality. In this study, egg weight was significantly improved and egg breaking rate was reduced in the treatment group, suggesting the GMB has a more pronounced effect on improving the value and quality of eggs.

The presence of serum enzymes and their levels in serum can provide some indication of the extent of organ or tissue damage. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin are important indicators for evaluating the liver function of poultry because they are synthesized in the liver. Dietary supplementation of coated sodium butyrate could significantly reduce serum ALT and total bilirubin contents and serum AST contents of 44-week-old black-bone breeder hens at 36 weeks of age (Xing, 2017). Oral administration of tributyrin can significantly decrease the levels of serum alanine aminotransferase, aspartate aminotransferase, and total bilirubin in rats with liver injury caused by endotoxin (Miyoshi et al., 2011). This is consistent with the results found in our study that dietary supplementation with 500 mg/kg monobutyrin can significantly reduce the content of aspartate aminotransferase and total bilirubin, as well as the trend of decreasing alanine aminotransferase. However, sodium butyrate supplementation did not significantly reduce aspartate aminotransferase in serum, but alanine aminotransferase decreased (Elnesr et al., 2019). This study and previous studies have shown that butyrin can reduce alanine aminotransferase in host animals, but the mechanism of action is not clear. Supplementation with butyrin has been reported to reduce serum triglyceride and total cholesterol concentrations (Yang et al., 2018). Broilers supplemented with a mixture of monobutyric acid and tributyric acid had lower serum cholesterol levels compared to controls (Bedford et al., 2017). However, these results were not observed in our study. Reasons for different results may be related to the form of butyric acid derivative supplementation, dosage, environmental factors, and animal species.

Antioxidant processes in animal bodies are critical to improving animal health, growth, production, and economic benefits. T-SOD and GSH-PX are important antioxidant enzymes in organisms, which play an important role in scavenging superoxide radicals, and peroxides and preventing or reducing the formation of hydroxyl radicals. T-AOC is a comprehensive index to measure the body's antioxidant function, and the level of MDA content reflects the degree of lipid peroxidation mediated by oxygen free radicals. The supplementation with sodium butyrate can significantly increase the serum T-SOD, GSH-PX, and catalase, and reduce the content of MDA in broilers under hot conditions (Lan et al., 2020). Supplementation with tributyric acid can improve the T-AOC of broiler breeder ovaries and most parts, and reduce MDA, which may also be the possible reason for the increase of the effect of tributyric acid on protein quality (Wang et al., 2021). Likewise, previous studies have shown that supplementation with sodium butyrate increases T-SOD activity and reduces MDA levels in breeders (Alhaj et al., 2018). In our study, we observed that serum T-AOC and T-SOD were also significantly increased by GMB while MDA and GSH-PX did not differ significantly, suggesting that GMB could more effectively protect laying hens from oxidative damage.

There is increasing evidence that animal performance is closely related to the modulation of gut microbiota by feed supplementation (Pan and Yu, 2014). Relatively few studies have been done on the effect of glycerol monobutyrate supplementation on the cecal microbiota. Yang et al. (2018) noted that supplementation with 3,000 ppm butyrate altered the gut microbiota. Alpha diversity index and microbiota composition at the phylum level were affected by dietary supplementation with unprotected butyrate (Moquet, 2018). In the present study, alpha diversity indices including Ace, Chao1, Shannon, Simpson, goods_coverage, and PD_whole tree were not affected by glycerol monobutyrate supplementation. The principal coordinates analysis chart shows that the samples in the control group and the GMB group are relatively densely clustered, which suggests that there is not much difference between the two groups of bacteria. One of the possible reasons for the bacteriostatic or bactericidal effect of butyrate is that the dissociation of SCFAs in the bacterial cytoplasm disrupts the proton motive force across the membrane and reduces the pH of the cytoplasm (Moquet, 2018). *Proteobacteria* mainly consists of many gram-negative bacteria such as *Vibrio cholerae*, *Helicobacter pylori*, *Salmonella*, and *Escherichia coli*, which could result in diarrhea, gastritis, vomiting, gastrointestinal ulcers, and even death, posing a great threat to animal health (Liu et al., 2021). Most researchers' research on butyrin has focused on its antibacterial and bacteriostatic effects, such as on *Salmonella* and *Escherichia coli*. The small chain fatty acid butyrate has been reported to reduce *Salmonella* (Rebollada-Merino et al., 2020). Zhang et al. (2011) reported that sodium butyrate prevents growth reduction in birds challenged by Salmonella Enteritidis. Metabolic disorders of gut microbiota are often accompanied by an increase in *Proteobacteria* (Shin et al., 2015). Overall, although the caecal microbiota composition did not change much, the *Proteobacteria* and *Enterobacter* were significantly less affected by GMB. This suggests that GMB may maintain or even improve the homeostasis of the cecal microbial system by reducing the relative abundance of *Proteobacteria* and *Enterobacter*. One of the potential mechanisms by which the significant reduction of harmful bacteria-enriched *Proteobacteria* may improve egg quality.

CONCLUSIONS

This study showed that dietary supplementation with 500 mg/kg of GMB improved egg weight, eggshell quality, serum antioxidant capacity, and reduced egg breaking rate in late laying hens. In the cecal microbiota, GMB supplementation had no effect on the composition of the microbiota and the Alpha diversity index except that it significantly reduced the relative abundance of *Proteobacteria* and *Enterobacter*. These findings shed new light on the use of GMB as a functional ingredient to improve the production cycle and egg quality of laying hens, which has important implications for the healthy development of the laying hen industry. However, the mechanism and optimal ratio of GMB need to be further explored.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethical Committee and conducted under the supervision of the Institutional Animal Care and Use Committee of Foshan University (Foshan, China).

AUTHOR CONTRIBUTIONS

HZ designed the experiment. GX and LG finished the statistical analysis of all data and the original draft written. LZ and YY conducted the animal feeding and the sample analysis. XY and QQ participated in the sample collection. All authors read and approved the final manuscript.

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