Effects of melittin on laying performance and intestinal barrier function of quails

Zhili Li, Rongxu Liu, Xuehan Wang, Haigang Wu, Xianguo Yi, Li Huang, and Qingming Qin

Engineering and Technology Research Center for Waterfowl Resources Development and Utilization and Epidemic Disease Prevention and Control of Henan Province, Xinyang Agriculture and Forestry University, Xinyang, Henan Province, PR China

ABSTRACT To study the effects of melittin on egg-laying performance and intestinal barrier of quails, 240 quails (aged 70 d) were randomly divided into 4 groups with 6 replicates (10 quails per replicate). They were fed with basal diet (group B), basal diet + 0.08 g/kg melittin (group BA1), basal diet + 0.12 g/kg melittin (group BA2) and basal diet + 0.16 g/kg melittin (group BA3). The experiment lasted for 21 days. The eggs were collected every day. At the end of the experiment, duodenal, jejunal, and ileal tissues were collected, and the cecal contents were sampled. Intestinal antioxidant index, barrier function, and intestinal flora were analyzed. The results showed that the addition of melittin significantly increased the laying rate and average egg weight. Addition of melittin significantly increased the antioxidant function, mechanical barrier, immune barrier, and villus height to crypt depth ratio of small intestine. Addition of melittin had no significant effect on the α and β diversity of cecal flora, but significantly increased the abundance of Bacteroidales at family level and genus level. Bioinformatics analysis of cecal content showed significant increase in COG functional category of cytoskeleton, and significant decrease in RNA processing and modification in group BA2. KEGG functional analysis showed significant decrease in steroid biosynthesis, caffeine metabolism, and cytochrome P450 pathways in group BA2. In conclusion, addition of 0.12 g/kg melittin to feed improved the laying performance and the intestinal antioxidant capacity and barrier function of quails but had no significant effect on the composition and structure of cecal microbial community. This study provides experimental data and theoretical basis for the application of melittin as a new quail feed additive.

Key words: melittin, quail, laying performance, intestinal barrier, antioxidation

INTRODUCTION Maintaining animal intestinal health is the key to achieve maximum productivity and safety of products. Large-scale breeding is mostly used in poultry production, and as the scale of farming and the density of rearing increases, it also increases the risk of transmission of infectious diseases by air and body fluids. The knowledge that antibiotics can prevent disease and promote growth fueled widespread use of antibiotics in the poultry and livestock industry (Van Boeckel et al., 2015). Antibiotics are even used in the treatment of viral respiratory tract infections in livestock and poultry (Rosini et al., 2020). However, antibiotic abuse leads to the imbalance of intestinal flora in livestock and poultry leading to emergence of drug-resistant pathogens and reduces the protective effect of microflora on pathogen invasion (Marshall and Levy, 2011; Chang et al., 2015). It also has a negative impact on the immune response, making individuals more vulnerable to infection (Sansone et al., 2015; Rosini et al., 2020). This represents a serious public health problem and causes serious complications, such as infection, allergies, abnormal brain development, and autoimmune diseases (Sola, 2020). Consequently, considerable research is underway to identify better alternatives to antibiotics for livestock and poultry.

The term antimicrobial peptides (AMP) refers to a kind of important defense molecules against the invasion of pathogenic microorganisms. AMP widely exist in the biological world and are an important component of biological innate immunity. These exhibit a broad range of biological activities such as antibacterial, antiviral, antifungal, antiparasitic, and anti-tumor activities; moreover, these are not vulnerable to the phenomenon of emergence of drug resistance (Regmi et al., 2016). Currently, indiscriminate prophylactic use of antibiotics in the farming industry is gradually being banned, and no new antibiotic substitutes have been developed. Therefore, research and
development of AMP for use in place of antibiotics as new feed additives is of great economic benefit and far-reaching significance. Melittin is an insect AMP, a cationic polypeptide composed of 26 amino acids (Jamasi et al., 2014). Under normal physiological conditions, the peptide has 4 + charges at the N terminal and 2 + charges at the C terminal, a total of 6 + charges (Raghuraman and Chattopadhyay, 2007). Melittin forms a monomer α-helix when it binds to the lipid bilayer of the cell membrane, which enables its penetration in the cell membrane and allows it to act on the cell substructure at the molecular level (Othon et al., 2009). Recent studies have demonstrated bactericidal activity of melittin against sensitive and drug-resistant bacterial strains (Dosler and Gerecker, 2012; Choi et al., 2015; Memariani et al., 2019; Pashaei et al., 2019; Lima et al., 2021). However, the effect of melittin on intestinal health is not clear. In-depth characterization of the effect of melittin on intestinal health will enable a better understanding of its potential role in maintaining intestinal health for prevention of pathogen infection.

Therefore, the experimental model used in this study was quail. Different concentrations of melittin were added to the feed to investigate the effect of melittin on intestinal immune function, barrier function, and intestinal flora of quails. The objective was to provide experimental data for the potential application of melittin as a substitute for antibiotics.

**MATERIALS AND METHODS**

**Experimental Animals, Diets, and Experimental Design**

A total of 240 (70-day-old) female African black quails were purchased from a quail breeding farm in Pingdingshan City, Henan Province. After 14 d of adaptation, quails were randomly divided into four groups: control group (group B, basal diet), group BA1 (basal diet supplemented with melittin, 0.08 g/kg), group BA2 (basal diet supplemented with melittin, 0.12 g/kg), and group BA3 (basal diet supplemented with melittin, 0.16 g/kg). There were 6 replicates in each group and 10 quails in each group. Melittin is a polypeptide product (activity ≥ 1 MIU/g, Item No. 361001) obtained by co-fusion expression genetic engineering technology, which was donated by Guangdong Henmao Biological Agricultural Technology Co. Ltd, Guangdong, China. We followed the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of People’s Republic of China for all our experimental procedures. In this experiment, the basic diet of quails was corn-soybean meal diet. Analysis of feed raw material composition and nutritional composition is shown in Supplementary Table 1. The experiment lasted for 21 d.

In this experiment, quails were raised in cages (0.50 m × 0.55 m × 0.45 m, 5 quails per cage) in a controlled environment (temperature 24 ± 2°C; relative humidity 50%–60%, light duration: 16 h) and provided ad libitum access to drinking water. Melittin was dissolved in the water, sprayed on the feed, and uniformly mixed.

**Laying Performance**

Feed intake and leftover feed, egg production and egg weight were recorded every day. At the end of the experiment, the average daily feed intake (ADFI), laying rate, average egg weight, and feed-egg ratio were calculated.

**Sample Collection**

After the 21st day of the experiment, one quail was randomly selected for sampling from each group. Quails were sacrificed by cervical dislocation. The contents of quail cecum were collected aseptically and immediately frozen in liquid nitrogen for analysis of intestinal flora composition. The tissues of duodenum, jejunum and ileum (approximately 2 cm) were collected, rinsed with normal saline, frozen immediately in liquid nitrogen, and stored in a refrigerator at −80°C. All enteric intestinal tissues were fixed with 4% paraformaldehyde.

**Intestinal Histology**

The intestinal samples fixed by paraformaldehyde were dehydrated, transparent, waxed, paraffin-embedded, sliced, and stained according to Iji et al. (2001). NikonNi-U microscope was used for histological examination and photography. Villus height (VH) and crypt depth (CD) were measured, and the ratio of villus height to crypt depth (VH/CD) was calculated.

**Detection of Antioxidant Indices in Intestinal Tissue**

The contents of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) in duodenum, jejunum, and ileum were detected by a commercially available kit (Sino Best Biological Technology Co. Ltd.), as per the enclosed instructions.

**Detection of Intestinal Immune Index and Mechanical Barrier Gene Expression**

The IgA content in duodenum, jejunum, and ileum was detected using an ELISA kit (Sino Best Biological Technology Co. Ltd., China), according to the manufacturer’s instructions.

Total RNA was extracted from tissues according to the instructions of RNA extract (Wuhan Servicebio Technology Co. Ltd., China), and the concentration of tissue RNA was determined using ultra-micro ultraviolet spectrophotometer (NanoDrop2000, ThermoFisher Scientific Co. Ltd., USA). According to the instructions of reverse transcription kit (Wuhan Servicebio Technology Co. Ltd.,
Bioinformatics Analysis of Intestinal Flora

The cecal contents were subjected to high-throughput sequencing by Shanghai Origin gene Bio-pharm Technology Co. Ltd., China. The variable region of bacterial V3-V4 was amplified by PCR using 16s rRNA gene 515F (5′-GTGCCAGCMGCCGCGGTAAUT-3′) and 806R (5′-GGAATACHVGGGTGTCTTAATMUT-3′). An Illumina HiSeq 2500 sequencing platform was used for high-throughput DNA sequencing, and QIME V1.9.0 and FLASH software packages were used for screening and assembly of the 16S data sequences. The high-quality sequences were compared with the Silva reference database (https://www.arb-silva.de/), and the UCLUST algorithm (http://drive5.com/usearch/Manual/uclust_algo.html) was used for classifying into OTU with 97% similarity level; the RDP classifier Bayesian algorithm was used to classify the OTU representative sequences. The functional genomes of all samples were predicted using PICRUSt v1.1.327 (Langille et al., 2013). STAMP 2.1.3 t test was used to compare the functional differences among groups (Maeda et al., 2020).

Statistical Analysis

The linear mixed model in SPSS Statistics 26 (SPSSS Inc. Chicago, IL) was used for statistical analysis, and GraphPad Prism 8 software (GraphPad Software, San Diego, CA) was used for generating graphs. The treatment group and the sampling time were designated as fixed factors and quail identity as the random factor. The means were compared by LSD method. The QIIME v1.9.0 was used to analyze the diversity differences of α and β, and R v.2.15.3 was used to present the results.

RESULTS AND

Laying Performance

As shown in Table 1, melittin significantly (P < 0.05) increased ADFI, laying rate increased, and average egg weight. However, melittin significantly (P < 0.05) decreased feed-egg ratio in group BA1 and BA2, and increased (P < 0.05) significantly in group BA3.

Small Intestinal Histomorphology

As shown in Table 2, melittin significantly (P < 0.05) increased VH and VH/CD in duodenum and VH/CD in jejunum, and significantly (P < 0.05) decreased CD in duodenum, jejunum and ileum.

Antioxidant Function of Small Intestine

When three different doses of melittin were added to the diet, 0.12 g/kg had the best effect on the antioxidant function of quails, which significantly increased (P < 0.05) the activities of CAT, SOD, and GSH-Px in duodenum, jejunum and ileum; however, only some antioxidant indices were increased in the other two groups. The MDA content in small intestine decreased significantly in the addition group (P < 0.05) (Table 3).

Small Intestinal Mechanical Barrier Gene

The effect of melittin on intestinal mechanical barrier gene in quails is shown in Figure 1. Melittin significantly increased the relative expression of Claudin-1 and zonula occludens 1 (ZO-1) mRNA in jejunum and ileum in group BA1 were increased significantly (P < 0.05); the relative expressions of ZO-1, Occludin, and Claudin-1 in duodenum, Mucin-2, ZO-1, Occludin, and Claudin-1 in jejunum and Mucin-2 mRNA in ileum were significantly increased in group BA2 (P < 0.05). The relative expression of Mucin-2 mRNA in duodenum in group BA3 was significantly lower than that in group B (P < 0.05).

Immune Function of Small Intestine

As shown in Figure 2A, B and C, melittin significantly up-regulated the expression of IL-2 in ileum, IFN-γ in duodenum and TLR-4 mRNA in jejunum in group BA1 (P < 0.05); the relative expressions of IL-2, IFN-γ, and TLR-4 in duodenum, IFN-γ and TLR-4 in jejunum, and IL-2 and TLR-4 mRNA in ileum were significantly increased in group BA2 (P < 0.05). Melittin significantly down-regulated the expression of IFN-γ mRNA in ileum in group BA2 and BA3 (P < 0.05) (Figures 2B). Melittin significantly up-regulated the IgA content in jejunum and ileum in groups BA1 and BA2 (P < 0.05) (Figure 2D).

Cecal Microbiological Analysis

Alpha Diversity Analysis There was no significant difference between group B and group BA2 with respect to ace, chao, shannon, or simpson indices (P > 0.05) (Figure 3).

β Diversity Analysis Figure 4A shows similar dispersion of the two groups of samples, and there is no obvious aggregation between the groups. In addition, the straight-line distance of each sample on the 2-dimensional diagram is similar, and there is no significant difference between the two groups. Figure 4B shows no
difference in the composition of bacterial communities among the samples.

**Community Composition Analysis of Flora** Analysis of community composition can help identify the kind of microorganisms in the sample and the relative abundance of each microorganism. At the phylum level (Figure 5A), the intestinal flora of the two groups were mainly composed of *Bacteroidetes, Firmicutes* and *Actinobacteria*. At the genus level (Figure 5B), the dominant bacteria of the top 10 species were mainly *Bacteroides*, *Faecalibacterium*, *Ruminococcus*, and *Alistipes*. There was no significant difference in the relative abundance of dominant flora at gate level between group BA2 and group B ($P > 0.05$) (Figure 6A), but *Bacteroides* at family level and genus level was significantly increased ($P < 0.05$) (Figure 6B and C).

### Table 1. Effects of melittin on average daily feed intake, laying rate, average egg weight, and feed to egg rate of quails.

| Item                        | B$^1$ | BA1 | BA2 | BA3 | SEM | F-value | P-value |
|-----------------------------|-------|-----|-----|-----|-----|---------|---------|
| ADFI (g/d)                  | 25.19b| 25.77a,b| 26.61a| 26.33a| 0.13 | 5.75    | 0.0304  |
| Laying rate (%)             | 85.32b| 86.51b| 88.17a| 85.24b| 0.27 | 6.42    | 0.0003  |
| Average egg weight (g)      | 10.93b| 11.44a | 11.83a| 10.95a| 0.04 | 151.32  | 2.70×10⁻⁵|
| Feed to egg rate            | 2.70b | 2.61c | 2.55a | 2.82a | 0.02 | 21.53   | 0.0004  |

$^a$,$^b$, $^c$In the same row, values with different letter superscripts indicate significant difference ($P < 0.05$), while those with the same or no letter superscripts indicate no significant difference ($P > 0.05$).

1Group B: control group; group BA1: 0.08 g melittin/kg of diet; group BA2: 0.12 g melittin/kg of diet; group BA3: 0.16 g melittin/kg of diet. Abbreviations: ADFI, average daily feed intake.

### Table 2. Effect of melittin on intestinal villus height, crypt depth, the ratio of villus height to crypt depth of quails.

| Item                        | B$^1$ | BA1 | BA2 | BA3 | SEM | F-value | P-value |
|-----------------------------|-------|-----|-----|-----|-----|---------|---------|
| Duodenum                    |       |     |     |     |     |         |         |
| VH (μm)                     | 673.29b| 738.44a | 764.75a| 706.33b | 12.27 | 5.04    | 0.0269  |
| CD (μm)                     | 66.19a | 60.04b | 53.49b | 57.83a | 1.45 | 4.84    | 0.0037  |
| VH/CD                       | 10.82a | 12.77a | 14.72a | 12.56a | 0.39 | 22.65   | 0.0011  |
| Jejunum                     |       |     |     |     |     |         |         |
| VH (μm)                     | 383.83| 390.08| 444.17| 395.66| 12.44 | 1.79    | 0.2370  |
| CD (μm)                     | 47.70b| 40.09c| 38.34c | 43.16c | 0.83 | 63.72   | 1.74×10⁻⁷|
| VH/CD                       | 8.27b | 9.79b | 11.72a | 9.36b | 0.36 | 6.69    | 0.0165  |
| Ileum                       |       |     |     |     |     |         |         |
| VH (μm)                     | 374.48| 399.52| 412.37| 385.85| 9.58 | 31.94   | 5.70×10⁻⁵|
| CD (μm)                     | 46.15c| 41.37b| 40.27b | 42.97a | 0.72 | 4.80    | 0.0330  |
| VH/CD                       | 8.41a | 9.84  | 10.35 | 9.47  | 0.32 | 1.83    | 0.2264  |

$^a$,$^b$, $^c$In the same row, values with different letter superscripts indicate significant difference ($P < 0.05$), while those with the same or no letter superscripts indicate no significant difference ($P > 0.05$).

1Group B: control group; group BA1: 0.08 g melittin/kg of diet; group BA2: 0.12 g melittin/kg of diet; group BA3: 0.16 g melittin/kg of diet. Abbreviations: VH, villus height; CD, crypt depth; VH/CD, the ratio of villus height to crypt depth.

### Table 3. Effect of melittin on intestinal catalase, superoxide dismutase, glutathione peroxidase, and malondialdehyde of quails.

| Item                        | B$^1$ | BA1 | BA2 | BA3 | SEM | F-value | P-value |
|-----------------------------|-------|-----|-----|-----|-----|---------|---------|
| Duodenum                    |       |     |     |     |     |         |         |
| CAT (U/g)                   | 123.94b| 153.13a,b| 173.23a| 78.17c | 8.59 | 31.94   | 5.70×10⁻⁵|
| SOD (U/g)                   | 247.24b| 291.66b| 292.24a | 250.13b | 6.88 | 10.53   | 0.0016  |
| GSH-Px (nmol/min/g)         | 42.66b | 45.56a | 51.97a | 43.19b | 1.01 | 8.97    | 0.0013  |
| MDA (nmol/g)                | 25.36a | 13.38c | 13.30c | 18.90b | 1.24 | 23.31   | 3.10×10⁻⁵|
| Jejunum                     |       |     |     |     |     |         |         |
| CAT (U/g)                   | 135.33b| 155.46b| 228.76b | 135.52c | 8.62 | 42.53   | 7.97×10⁻⁶|
| SOD (U/g)                   | 117.78b| 155.95a | 157.28a | 148.90a | 4.34 | 10.88   | 3.70×10⁻⁵|
| GSH-Px (nmol/min/g)         | 36.67c | 41.41b,c| 50.64a | 45.35b | 1.32 | 18.76   | 0.0001  |
| MDA (nmol/g)                | 28.24a | 14.39b | 17.24b | 15.55b | 1.49 | 8.08    | 0.0058  |
| Ileum                       |       |     |     |     |     |         |         |
| CAT (U/g)                   | 114.90c | 239.25a | 273.87a | 157.36b | 14.40 | 46.73   | 3.00×10⁻⁶|
| SOD (U/g)                   | 147.19c | 152.05c | 40.32b | 530.41a | 35.15 | 578.53  | 7.32×10⁻¹⁴|
| GSH-Px (nmol/min/g)         | 37.62c | 43.24a | 49.52a | 35.90a | 1.46 | 10.45   | 0.0035  |
| MDA (nmol/g)                | 65.07b | 17.41a | 29.65b | 31.33b | 3.94 | 25.90   | 0.0001  |

$^a$,$^b$, $^c$In the same row, values with different letter superscripts indicate significant difference ($P < 0.05$), while those with the same or no letter superscripts indicate no significant difference ($P > 0.05$).

1Group B: control group; group BA1: 0.08 g melittin/kg of diet; group BA2: 0.12 g melittin/kg of diet; group BA3: 0.16 g melittin/kg of diet. Abbreviations: CAT, catalase; df, degree of freedom; GSH-Px, glutathione peroxidase; MDA, malondialdehyde; SOD, superoxide dismutase.
Figure 1. Effect of melittin on intestinal mechanical barrier of quail. The same letter superscript indicates no significant difference \((P > 0.05)\), whereas different letter superscript indicates significant differences \((P < 0.05)\). Mean ± standard error of the mean (SEM) values from six independent experiments are presented. Group B: control group; group BA1: 0.08 g melittin/kg of diet; group BA2: 0.12 g melittin/kg of diet; group BA3: 0.16 g melittin/kg of diet. ZO-1, zonula occludens 1.

Figure 2. Effects of melittin on IL-2, IFN-\(\gamma\), TLR-4, and IgA in small intestine of quail. The same letter superscript indicates no significant difference \((P > 0.05)\), whereas the different letter superscript indicates significant differences \((P < 0.05)\). Mean ± standard error of the mean (SEM) values from six independent experiments are presented. Group B: control group; group BA1: 0.08 g melittin/kg of diet; group BA2: 0.12 g melittin/kg of diet; group BA3: 0.16 g melittin/kg of diet. IL-2, interleukin-2; IFN-\(\gamma\), interferon-\(\gamma\); TLR-4, Toll-like receptor 4; IgA, immunoglobulin A.
**Function Abundance Profile of Cecal Microbiota**

Based on the 16s function prediction of PICRUSt analysis platform, the OTU abundance table obtained was compared with Greengene database, and the corresponding information about Clusters of Orthologous Groups (COG) functional categories and KEGG pathways were obtained. The prediction results of COG function showed that cytoskeleton was significantly increased and RNA processing and modification was significantly decreased in group BA2 ($P < 0.05$) (Figure 7A). The results of KEGG function prediction showed that Melittin significantly ($P < 0.05$) downregulated steroid biosynthesis, caffeine metabolism, and cytochrome P450 pathways in group BA2 (Figure 7B).

**DISCUSSION**

Melittin is the main pharmacologically active component in bee venom, and it is a promising candidate for tumor therapy. This preparation has been shown to regulate multiple anticancer mechanisms in preclinical cell culture and animal models (Rady et al., 2017). Previous studies have demonstrated the biological effects of melittin against bacteria, fungi, viruses, and parasites (El-Seedi et al., 2020; Memariani and Memariani, 2020; Memariani and Memariani, 2021). In this study, we demonstrated for the first time that melittin can improve quail production performance, and improve intestinal immune barrier and mechanical barrier. PCA analysis and NMDS analysis showed no significant in clustering between 0.12 g/kg group and control group, which could significantly increase the abundance of Bacteroidales at family level and genus level.

A few studies have investigated the effect of melittin on animal production performance, but only in rabbits (Elkomy et al., 2021) and chickens (Rabie et al., 2018). Melittin was found to significantly decrease the average daily feed intake of broilers, while there was no significant effect in rabbits; however, it was found to improve the reproductive ability of rabbits. The results showed that the addition of melittin significantly increased the average daily feed intake, egg laying rate, and average egg weight, and decreased the feed-egg ratio of quails. Previous studies have shown that both porcine intestinal AMP (Wang et al., 2009) and AMP Plectasin (Zhang et al., 2021) can significantly increase the average daily feed intake of chickens. Currently, there is a paucity of studies on the effect of AMP on poultry laying performance. Compared with the control group, the addition of AMP cecropin significantly increased the laying rate and feed-egg ratio, but it had no significant effect on the average daily feed intake or average egg weight (Chen et al., 2020).

**Figure 3.** Effect of melittin on microbial α diversity in cecum of quail. Group B: control group; group BA2: 0.12 g melittin/kg of diet.

**Figure 4.** Effect of melittin on microbial β diversity in cecum of quail. (A): PCA analysis; (B): NMDS analysis based on UniFrac. Group B: control group; Group BA2: 0.12 g melittin/kg of diet.
The small intestine is the main site for nutrient absorption and transport (Xie et al., 2020), and its morphology and growth performance are closely related to intestinal health (Ao and Kim, 2020). VH/CD ratio is an important marker of intestinal health status of birds (Jia et al., 2010; Jayaraman et al., 2013). The increase in VH/CD ratio is associated with enhanced digestion and absorption, and accelerated growth (Zhang et al., 2020). Some studies have demonstrated a beneficial effect of AMP on intestinal morphology. Dai et al. (2021) reported that the AMP MicrocinC7 significantly increased the villus height, decreased the crypt depth, and increased the VH/CD ratio of the small intestine in broilers. According to Choi et al. (2013), the villus height of duodenum, jejunum, and ileum showed a linear increase with the addition of antimicrobial peptide-A3 in diet. Consistent with previous studies, in the present study, melittin increased the villus height and VH/CD ratio and decreased the crypt depth of *intestinum tenue* in quails. These results show that melittin can protect and promote the repair of intestinal villi, which is beneficial to the growth of intestinal epithelial cells. The consequent increase in the absorption area of nutrients improves the efficiency of nutrient absorption and promotes the synthesis of important substances.

**Figure 5.** Effect of melittin on the relative abundance of intestinal microorganisms in quails. (A): relative abundance distribution of flora at phylum level; (B): relative abundance distribution of flora at genus level. Group B: control group; Group BA2: 0.12 g melittin/kg of diet.
Figure 6. Comparative analysis of the abundance of dominant cecal microflora at phylum level between group B and group BA2 quails. (A): difference analysis of phylum level flora abundance; (B): difference analysis of family level flora abundance; (C): difference analysis of genus level flora abundance. Welch’s t test bar plot on Phylum, Family and Genus level. B, control group; BA2, 0.12 g/kg group. P-value is represented by the right ordinate, while family name is represented by the left ordinate.

Figure 7. Effects of melittin on functional information and abundance of microorganisms COG and KEGG in cecum of quail. (A) and (B) show the predicted abundance information of COG functional categories and KEGG pathways, respectively. Group B: control group; Group BA2: 0.12 g melittin/kg of diet.
Antioxidant enzymes (including SOD, CAT, and GSH-Px) represent the first line of defense in animals and can scavenge free radicals produced by oxidative metabolism (Xie et al., 2020). The MDA level indirectly reflects the degree of oxidative damage (Mishra and Jha, 2019). In a previous study, melittin was found to significantly increase the serum SOD and GSH-Px content and decrease the MDA content in rabbits (Elkomy et al., 2021). In this study, melittin (0.12 g/kg) significantly increased the activities of SOD, CAT, and GSH-Px in the small intestine of quails, and significantly decreased the concentration of MDA. These findings suggest that melittin can improve the antioxidant capacity of quail small intestine, protect cells from oxidative damage, and play an important role in stabilizing intestinal health.

A normal intestinal mucosal barrier has four components: mechanical, chemical, immune, and biological barrier, which can resist harmful substances entering the body. The main components of mechanical barrier include mucin secreted by the intestinal epithelium and the tight junction (TJ) of intestinal epithelial cells. Mucin can separate the internal environment from the intestinal environment, and mucin-2 is the main component of intestinal mucus which acts as a surface cleaner, antagonizes the adhesion and invasion of pathogenic bacteria, and plays an important role in intestinal microecological balance (Barbara et al., 2021). The TJs control the accessory pathway permeability of intestinal cells, and the damage of TJ and the abnormal expression of TJ-related genes can increase the pericellular permeability to intraluminal antigens, inflammatory factors, and bacterial translocation, resulting in persistent inflammation, tissue damage, and reduced nutritional retention (Suzuki, 2013). At the same time, TJ plays a vital role in resisting oxidative stress products (Tan et al., 2018). In the present study, addition of melittin to the feed, especially 0.12 g/kg, increased the relative expression of ZO-1, Occludin, Claudin-1, and mucin-2 mRNA in *intestinum tenue*. Addition of 0.16 g/kg melittin decreased the relative expression of Mucin-2, Claudin-1, Occludin, ZO-1 in duodenum and that of mucin-2, Claudin-1, Occludin mRNA in ileum. We speculate that this may be due to the high anti-inflammatory properties of 0.16 g/kg melittin and its irritant effect on the gastrointestinal tract. Mucin-2 is the first line of immune defense against pathogens. In previous studies, addition of mixed antimicrobial peptides (pectasin and cecropins) significantly increased the relative expression of Mucin-2 mRNA in jejunum of broilers (Xie et al., 2020), which was consistent with the results of this study. In addition, Dai et al. (2021) reported the same conclusion on connexin that the addition of Microcin C7 could significantly increase the relative expression of occludin and ZO-1 mRNA in jejunum of broilers.

Intestinal diseases of poultry can be effectively prevented and controlled by enhancing the intestinal immune function. Currently, functional amino acids (Jian et al., 2021), polysaccharides (Lv et al., 2021), probiotics (Bilal et al., 2021), and AMP (Dai et al., 2021; Zhang et al., 2021) are widely used to enhance the immune function of poultry.

IL-2 is a multipotent cytokine, which can induce killer cells to produce cytokines such as IFN-γ and TNF-α, which is necessary to maintain regulatory T cell (Treg) and immune homeostasis in the entire gastrointestinal tract (Zhou et al., 2019). In other studies, knockout of both IL-2 and IL-2R genes in mice was found to induce the development of inflammatory bowel disease (Sadlack et al., 1993; Hsu et al., 2009). Dietary supplementation of 0.12 g/kg melittin in the present study significantly increased the relative expression of IL-2 mRNA in duodenum and ileum. In the study by Chen et al. (2015), addition of MDPF significantly promoted the production of IL-2 by mouse splenocytes, which is consistent with our findings.

IFN-γ has antiviral, antitumor and immunomodulatory activities, and it is also a key regulator of immune cell activation (Gupta et al., 2020). IFN-γ also promotes the release of AMP and mucus from intestinal Paneth cells and goblet cells (Farin et al., 2014). In addition, IFN-γ plays an important role in the proliferation and apoptosis of intestinal epithelial cells by regulating the β-catenin signal pathway (Nava et al., 2010). Knockout of IFN-γ gene in mice abrogated the ability of macrophages to present antigens to immune cells, resulting in a significant decrease in the intensity of immune response mediated by intestinal mucosal cells (Jouanguy et al., 1999). In the present study, dietary melittin significantly increased duodenum and jejunum, and significantly decreased the relative expression of IFN-γ mRNA in ileum. In other studies, addition of mixed AMP was found to significantly increase the expression of IFN-γ mRNA in ileum of broilers (Xie et al., 2020). Addition of AMP MicrocinC7 reduced the expression level of IFN-γ mRNA in jejunum of broilers (Dai et al., 2021). Addition of pectasin significantly decreased the expression of IFN-γ mRNA in ileum of 21-day-old yellow-feathered chicks (Zhang et al., 2021). This inconsistency may be related to different AMP and species. Melittin has the opposite effect in different intestinal segments, which may be due to the different tolerance of different intestinal segments to melittin. Further studies are required to unravel the specific mechanism.

The earliest pattern recognition receptor is Toll-like receptor 4 (TLR4), which plays an important role in the innate immune system, mainly recognizing endotoxin/lipopolysaccharide (Medvedev, 2013). Recognition of LPS induces activation of the downstream signal pathway of TLR4, leading to lymphocyte activation, up-regulation/expression of stimulation signals, and the release of pro-inflammatory cytokines/chemokines (Kang et al., 2009). In a study by Ahmedy et al. (2020), melittin had no effect on TLR4 protein in mouse colon, but significantly reduced the TLR4 increased by ulcerative colitis. In the present study, addition of melittin up-regulated the expression of TLR4 mRNA in the whole small intestine. This result indicates that melittin activates TLR4 signal pathway, promotes the release of pro-inflammatory cytokines, increases intestinal epithelial permeability, and leads to the decrease of barrier function; however, our findings suggest that it significantly enhanced the intestinal barrier function. A previous
study suggested that in most cases, up-regulation of TLR4 expression may protect the intestinal tract (Fukata and Abreu, 2007), the specific mechanism of which is not clear.

Secretory IgA is the major component of the local immune barrier in the gut, which improves bactericidal ability by preventing pathogens from adhering to and penetrating the mucosal epithelium; in addition, it helps maintain symbiotic relationship with bacteria to provide immune defense (Mestecky and Meghee, 1987; Corthesy, 2013). The addition of porcine AMP was shown to promote the expression of secretory IgA in duodenum and jejunum of broilers (Bao et al., 2009) and small intestine of SPF chickens (Wang et al., 2009). In another study, AMP MicrocinC7 significantly increased the relative abundance of ileal secretory IgA in broilers (Dai et al., 2021). The results suggest that melittin can significantly increase the IgA content in jejunum and ileum. To summarize, melittin not only has bactericidal effect, but can also enhance the immune function of quail intestines.

Gastrointestinal microflora plays an important role in maintaining intestinal and nutritional health in poultry production (Wei et al., 2013). The intestinal microflora forms a protective layer by combining with the intestinal epithelial surface of intestinal cells (Yegani and Korver, 2008), which protects the host from pathogens. Altering the composition of intestinal flora has been shown to cause intestinal inflammation (Khalif et al., 2005; Deriu et al., 2016; Mima et al., 2017). In the present study, 0.12 g/kg melittin had the best effect on intestinal health. In order to further study the effect on intestinal flora, we selected control group and 0.12g/kg melittin group for Illumina sequencing of 16S rRNA gene of flora. The results showed no significant effect of melittin on the microbial α diversity of quails. Thibodeau et al. (2015) showed that only extreme events that change the number of niches of different bacterial species can change α diversity. However, AMP were also reported to significantly affect the community composition and structure of cecal microorganisms in laying hens (Chen et al., 2020) and broilers (Xie et al., 2020). At the same time, in this study, there was no significant difference in the effect of melittin on the intestinal microflora β diversity of quails, which indicated similar colony composition between the control group and the melittin group.

We found that the intestinal flora of control group and melittin group were mainly composed of Bacteroidetes, Firmicutes, and Actinobacteria, and there was no significant difference between them. The Bacteroidales at family level and genus level in 0.12 g/kg melittin was significantly increased. Many studies have investigated the probiotic effect of Bacteroidales. Bacterodes were shown to play an important role in helping the host to decompose polysaccharides to improve the utilization rate of nutrients (Bücked et al., 2004), promoting the development of the host immune system (Stappenbeck et al., 2002; Hooper, 2004), and maintaining the balance of intestinal microbiology (Hooper et al., 2001; Sears, 2005).

Intestinal microorganisms and their metabolites not only affect the development of host intestinal immune system, the repair and renewal of intestinal epithelial tissue, and the maintenance of intestinal function, but also affect the expression of host genes through their own biological responses and functions, thus participating in the metabolism and regulation of intestinal nutrients (Bücked et al., 2004; Qiao et al., 2014). Through the gene annotation of quail intestinal microflora by PICRUSt system, we found that melittin significantly increased cytoskeleton, as per the predicted COG functional categories. This further indicated that melittin improved the intestinal mechanical barrier, because the cytoskeleton forms intestinal tight junction protein complexes with four transmembrane proteins (Occludin, Claudins, tricellulin, and JAM) and cytoplasmic molecules such as ZOs and cingulin (Bazzoni et al., 2000). In the prediction of KEGG function, melittin significantly decreased steroid biosynthesis, caffeine metabolism, and cytochrome P450 pathways. However, there was little enrichment in each pathway, and the effect of these changes on the body needs to be further studied.

CONCLUSIONS

In this study, supplementation of 0.12 g/kg melittin to the basic diet of quails was found to improve egg laying performance, intestinal antioxidant function, mechanical barrier, and immune function. Melittin had no significant effect on α and β diversity of cecal flora, but significantly increased the abundance of Bacteroidales at family level and genus level. To some extent, melittin had an impact on the COG functional categories and KEGG pathways. These results are of great significance to promote the intestinal health of quails and provide a theoretical basis for the application of melittin in poultry breeding.

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DISCLOSURES

The authors declare that there are no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.102355.
Ahmedy, O. A., S. M. Ibrahim, H. H. Salem, and E. A. Kandil. 2020. Antitumorogenic effect of melittin via mitigating TLR4/TRAF6 mediated NF-κB and p38MAPK pathways in acetic acid-induced ulcerative colitis in mice. Chem-Biol. Interact. 331:109276.

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