



Original Research Article

Marine red yeast supplementation improves laying performance by regulating small intestinal homeostasis in aging chickens



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ABSTRACT

Recent studies have shown that age-related aging evolution is accompanied by imbalances in intestinal homeostasis. Marine red yeast (MRY) is a functional probiotic that has been shown to have antioxidant, immune and other properties. Therefore, we chose 900 healthy Hy-Line Brown hens at 433 d old as the research subjects and evaluated the correlation between intestinal health, laying performance, and egg quality in aged hens through the supplementation of MRY. These laying hens were assigned into 5 groups and received diet supplementation with 0%, 0.5%, 1.0%, 1.5%, and 2% MRY for 12 weeks. The results showed that MRY supplementation increased egg production rate, average egg weight, and egg quality, and decreased feed conversion ratio and daily feed intake ($P < 0.05$). The MRY supplement improved antioxidant indicators such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), stimulated villus height, and increased the villus height to crypt depth ratio (V/C ratio) in the intestine ($P < 0.05$). It also regulated the expression of intestinal inflammatory factors (transforming growth factor- β [TGF- β], interleukin [IL]-1 β , IL-8, tumor necrosis factor- α [TNF- α]) while increasing serum immunoglobulin G (IgG) levels ($P < 0.05$). Furthermore, MRY supplementation upregulated the mRNA expression of tight junction proteins (occludin and zonula occludens-1 [ZO-1]), anti-apoptotic gene (*Bcl-2*), and autophagy-related proteins (beclin-1 and light chain 3I [LC3II]) in the intestine ($P < 0.05$). The MRY supplement also led to an increase in the concentration of short-chain fatty acids in the cecum, and the relative abundance of the phylum Bacteroidetes, and genera *Bacteroides* and *Rikenellaceae_RC9_gut_group*. The LEfSe analysis revealed an enrichment of *Sutterella* and *Akkermansia muciniphila*. In conclusion, the results of this experiment indicated that the additional supplementation of MRY can improve the production performance of laying hens and may contribute to the restoration and balance of intestinal homeostasis, which supports the application potential of MRY as a green and efficient feed additive for improving the laying performance in chickens.

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1. Introduction

In poultry production, the decline in egg production performance and egg quality resulting from the advancing age of chickens has caused significant economic losses to the poultry industry (Liu et al., 2013). Recently, there has been a new breeding objective of extending the laying period of hens and achieving 500 eggs in 100 weeks (Gautron et al., 2021; Liu et al., 2013). Some studies have

pointed out that in addition to disease infection, aging is another factor leading to decreased productivity of hens (Joyner et al., 1987; Molnár et al., 2016). Current research on aging in laying hens mostly focuses on ovarian oxidative stress (Lim and Luderer, 2011; Liu et al., 2018). However, studies in recent years have emphasized that the intestine is also a key target organ for improving the health of elderly animals and humans (Soenen et al., 2016), and the concept of “intestinal aging” has been proposed. Intestinal aging is related to the decline of intestinal function at the organ and cellular levels, including impairment of intestinal epithelial barrier function, immune dysfunction and intestinal dysbiosis (Buford, 2017; Branca et al., 2019), which are closely interconnected and collectively regulate the homeostasis of the intestinal tract. For example, increased intestinal permeability caused by biological polymorphism disorders can inhibit normal nutrient absorption, food metabolism and activate inflammatory responses (Clark et al., 2015). Increasing evidence has revealed that intestinal autophagy activity can maintain the balance of intestinal oxidative stress and play a role in reducing the damage to the intestinal barrier during the aging process (Gelino et al., 2016; Hansen et al., 2018). The disruption of intestinal homeostasis during aging may limit the optimal intake and utilization of nutrients, resulting in a decline in intestinal digestion and absorption, leading to the entry of pathogens, toxins and other harmful substances into the body through the intestines (Jing et al., 2014; Li et al., 2022). This further causes a series of problems such as decreased egg production performance of laying hens in the late laying period. Therefore, the dysregulation of intestinal homeostasis may potentially synergize with ovarian oxidative stress to hinder the economic efficiency of egg production in laying hens. Currently, dietary interventions such as natural plant phenolic compounds and fiber harbor anti-aging potential by regulating intestinal health (Henning et al., 2018; Wu et al., 2020). Probiotics have also exhibited the ability to improve age-related microbial dysbiosis and intestinal inflammation (Rawji et al., 2016).

Marine red yeast (MRY), a highly stress-resistant aquatic eukaryotic organism naturally found in the ocean (Yun et al., 2021), is an effective functional probiotic. It exhibits the ability to secrete various active metabolites, including carotenoids like β -carotene and astaxanthin, polysaccharides, and essential amino acids, making it highly biologically functional and nutritionally valuable for animals (Nutaratat et al., 2016). In recent years, the use of MRV as feed in the aquaculture of aquatic larvae has become increasingly widespread. Studies have reported that the addition of MRV to bait promotes the growth of aquatic animals, enhances their immunity, and improves their antioxidant capacity (Díaz et al., 2014; Paola and Dariel, 2014; Zhenming et al., 2006).

To the best of our knowledge, there are currently very limited studies on the effects of MRV on intestinal aging in laying hens during the late laying period, especially the correlation between intestinal health and egg production performance. Therefore, in this study, we evaluated the effects of MRV additives on the production performance and oxidative damage of laying hens in the late laying period, and further analyzed the intestinal structural properties, immune function, intestinal flora and aging. This study suggests that appropriately adding MRV to feed can improve the aging state of the intestine, thereby delaying the decline in laying performance in aged laying hens.

2. Materials and methods

2.1. Animal ethics statement

All procedures of this experiment have been approved by the Animal Care and Use Committee of Henan Agricultural University

(19-0068). The study reported in this paper complies with the ARRIVE guidelines.

2.2. Test materials

The MRV additive used in this test was a concentrated bacteria powder prepared by our laboratory. This product contained 6.67% MRV, 3.33% silicon dioxide, 20% water-soluble starch, and 70% anhydrous sodium sulfate. The experiment was conducted at Nanyang Fengyuan Poultry Industry Co., Ltd. (China, Henan), and the basal diet employed was the Laying Hen Compound Feed-526 produced by the company.

2.3. Experimental design and feeding management

We randomly divided healthy experimental animals (900 laying hens with no significant difference in egg production rate, $P > 0.05$) into 5 treatment groups. The control group (group 0) was fed a basal diet (Table 1), while the 4 experimental groups were fed the basal diet supplemented with 0.5%, 1.0%, 1.5%, and 2.0% MRV additives, respectively, for 12 weeks.

The contents of lysine, methionine, and cysteine in the feed were determined strictly in accordance with the Chinese National Standard (GB/T 18246-2019). The feed samples were hydrolyzed at 110 °C with 6 mol/L HCl for 24 h, and then a fully automated amino acid analyzer (L-8900, Hitachi, Tokyo, Japan) was used with a sodium citrate buffer solution as the mobile phase and ninhydrin for post-column derivatization. The nitrogen (N) content was determined using a fully automated Kjeldahl nitrogen analyzer (Kjeltec 8400, FOSS, Denmark) (Method, 2001.11; AOAC, 2005) by titration with 0.1 mol/L HCl, and the crude protein content in the samples was calculated ($N \times 6.25$). After treatment with concentrated hydrochloric acid and concentrated nitric acid, the calcium content was determined by titration with potassium permanganate solution (Method 927.06; AOAC, 2000). Based on the principle that molybdate solution can form a yellow complex with sulfuric acid or sulfate solution, the content of phosphorus in the feed samples was determined using a UV–Vis spectrophotometer (Cary 60, Agilent Technologies, California, USA) (Method 965.17; AOAC, 2000). The data provided by the Chinese Feed Database (2000) was used to determine the ME1, ME2, and ME3 values for corn, wheat, and soybean meal. The ME for this experiment was then calculated using the formula: $ME = \text{corn} \times ME1 + \text{wheat} \times ME2 + \text{soybean meal} \times ME3$.

The laying hens were housed in a three-tiered cage system (upper, middle, and lower tiers), with 6 replicates per group, 10 cages per replicate, and 3 chickens per cage, with feed and water ad libitum. Eggs were collected and weighed once a day at 10:00, and the remaining feed was weighed on a weekly basis. Routine immunization procedures were followed. At the end of the formal experimental period, the eggs collected on the final day were utilized for egg quality analysis. At the end of the experiment, 30 chickens (one from each replicate) were randomly selected from the control group and four MRV groups, and blood samples were collected to obtain serum. Euthanasia was performed by cervical dislocation, and liver tissues, cecal contents, as well as the duodenum, jejunum, and ileum tissues were collected for further analysis.

2.4. Egg production performance and egg quality

The weight, quantity, and mortality rate of each replicate egg were recorded daily. The feed consumption of each replicate was recorded weekly. Egg production rate, average daily egg weight, average daily feed intake (ADFI), and feed conversion ratio (FCR)

Table 1
Ingredients and nutrient level of the basal diet (air-dry basis).

Item	Dietary marine red yeast (MRY) additive content, %				
	0 (Control)	0.5	1.0	1.5	2.0
Ingredients, g/kg					
Corn	360.00	358.20	356.40	354.60	352.80
Wheat	350.00	348.25	346.50	344.75	343.00
Soybean meal	173.00	172.14	171.27	170.41	169.54
Calcium hydrogen phosphate	8.00	7.96	7.92	7.88	7.84
Stone powder	94.00	93.53	93.06	92.59	92.12
Premix ¹	15.00	14.93	14.85	14.78	14.70
Marine red yeast additive		5.00	10.00	15.00	20.00
Total	1000.00	1000.00	1000.00	1000.00	1000.00
Measured nutritional level, %					
ME, kcal/kg	2630	2630	2630	2630	2630
Crude protein	14.85	14.62	14.87	14.89	14.96
Calcium	3.57	3.47	3.45	3.48	3.47
Total phosphorus	0.51	0.52	0.52	0.51	0.51
Lys	0.78	0.78	0.78	0.78	0.78
Met	0.38	0.38	0.38	0.38	0.38
Met + Cys	0.63	0.63	0.63	0.63	0.63

ME = metabolizable energy.

¹ Premix provides per kilogram of feed: vitamin A, 10,000 IU; vitamin D₃, 3500 IU; vitamin E, 25 mg; vitamin K₃, 3 mg; vitamin B₁, 2 mg; vitamin B₂, 5 mg; vitamin B₆, 3 mg; vitamin B₁₂, 0.02 mg; niacin, 35 mg; D-calcium pantothenate, 15 mg; folic acid, 1 mg; biotin, 0.1 mg; choline, 200 mg; copper, 8 mg; iron, 40 mg; manganese, 100 mg; zinc, 80 mg; iodine, 1.2 mg; selenium, 0.3 mg; L-lysine, monohydrochloride, 2 g; DL-methionine, 1.5 g; salt, 3 g.

were calculated. The egg production rate was calculated as the average daily egg production per hen.

A total of 60 eggs were randomly selected from each group, with 10 eggs per replicate, for comprehensive assessments of internal and external quality. The eggshell thickness was measured using an eggshell thickness gauge (ETG-1061, Robotmation Co., Ltd., Japan), with measurements taken separately at the blunt end, middle, and pointed end of the egg, and the average of the three values was defined as the eggshell thickness. The eggs were placed on the eggshell strength tester (EFG-0503, Robotmation, Japan), and the pressure value displayed on the machine at the moment of eggshell fracture was recorded as the eggshell strength. The multifunctional egg quality analyzer (EMT-5200, Robotmation, Japan) was used to measure albumen height, yolk color, and Haugh units. Haugh units were calculated based on albumen height and egg weight using a simplified formula (Eisen et al., 1962): Haugh unit = $100 \log_{10} (H - 1.7W^{0.37} + 7.57)$, where H is the height of the egg white (mm), and W is the weight of the egg (g).

2.5. Determination of antioxidant index

The weight of each tissue was measured accurately to prepare 10% serum, liver, and yolk homogenate supernatant. To achieve this, each tissue was mixed based on the weight (g) to ice-cold physiological saline volume (mL) ratio of 1:9. Steel beads were added to facilitate thorough grinding using a grinder. The ground mixture was centrifuged at $3500 \times g$ for 10 min, and the supernatant was collected. This process was conducted at 4 °C. Specific measurements were performed according to the operating instructions provided by Nanjing Jian Cheng Bioengineering Research Institute (Nanjing, China) for the malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT) assay kits.

2.6. Intestinal morphology

The small intestine was isolated, and segments of the duodenum, jejunum, and ileum (approximately 1 cm each) were collected. After sterile PBS rinsing, the intestinal tissues were fixed in 4% paraformaldehyde and prepared for paraffin sectioning. The sections were stained with hematoxylin and eosin (H&E) and then

captured and measured using a Motic digital slide scanner and Motic DSA assistant Lite 1.0 software. Due to errors encountered during the fixation process of certain intestinal tissue fragments, only the intestinal samples from four chickens per group were selected (ensuring 6 representative fields of view for a specific intestinal segment of one chicken). Following the study by Sabour et al., (2019), villus height was defined as the vertical distance between the tip of the villus and the junction with the crypt. The depth of the invagination between adjacent crypts was referred to as crypt depth, and the villus height to crypt depth ratio (V/C ratio) was determined.

2.7. Determination of serum immunoglobulin content and intestinal inflammatory factors

Blood was collected in coagulation-promoting tubes and were centrifuged at 4 °C and $1500 \times g$ for 15 min to collect the serum. The intestinal tissue sample was added PBS and thoroughly homogenized for intestinal homogenate preparation. After centrifugation at approximately $3000 \times g$ for 20 min, the supernatant was collected. Enzyme-linked immunosorbent assay (ELISA) was performed according to the manufacturer's instructions provided by Yutong Biotechnology Company in Jiangsu, China.

2.8. Extraction of intestinal mRNA and analysis of gene expression

After grinding each replicate of frozen intestinal samples into a homogeneous paste, they were mixed thoroughly with Trizol reagent (10296028, Invitrogen, Carlsbad, CA, USA) for total RNA extraction. Subsequently, RNA samples with suitable concentration and purity were screened using 1% agarose gel electrophoresis and a Nano Drop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Primer design was carried out using Primers-BLAST in NCBI and synthesized by Beijing Qiangke Biotechnology Co., Ltd., China. The chicken β -actin gene served as an internal control, and the expression of certain genes in the intestine was calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2002). The primer sequences for quantitative reverse transcription polymerase chain reaction (qRT-PCR) are shown in Table 2. The reverse transcription reaction and cDNA synthesis

Table 2

Primer sets for quantitative real-time PCR.

Gene	GenBank number	Primer	Sequence of nucleotide (5' to 3')	Product size, bp
<i>IL-1β</i>	NM_204524.1	F	TGCCTGCAGAAGAAGCCTCG	204
		R	GACGGGCTCAAAAACCTCTCT	
<i>IL-8</i>	NM_205498.2	F	CTCTGTGCGCAAGGTAGGACG	240
		R	GCTGAGCCTTGGCCATAAGT	
<i>TNF-α</i>	XM_040647309.1	F	GCCCAGTTTCAGATGAGTTGCC	100
		R	AAGAGGCCACACACGACAG	
<i>TGF-β</i>	NM_205003.2	F	TGACCAGGGTAACAGGACCA	136
		R	ACGCCCAATCAACCGTTTTG	
Claudin-2	NM_001277622.1	F	CTCAGCCCTCCATCAAACA	82
		R	TGCTGCTGCTACACGTAT	
Occludin	XM_046904540.1	F	GCAGATGCTCAGCGTTACTAC	176
		R	CGAAGAAGCAGATGAGGCAGAG	
<i>ZO-1</i>	XM_046925214.1	F	CAAGGAGCCATTCCAGAGGG	125
		R	CCACACATTACCAAGGGGCT	
<i>ZO-2</i>	XM_046913144.1	F	TCACCTTCTTCTTCCCAATCT	178
		R	GCAACCTTTCCTCCATCTC	
<i>Bax</i>	XM_046922136.1	F	TCCTCATCGCCATGCTCAT	69
		R	CCTTGGTCTGGAAGCAGAAGA	
<i>Bak</i>	XM_046932962.1	F	ATGGATGCCTGTCTCTCTGTT	106
		R	GCAGAGCAGTCCAAAGACACTGA	
<i>Bcl-2</i>	XM_046910476.1	F	GATCGTCGCCTTCTTCGAGT	217
		R	GGCCTCATACTGTTGCCGTA	
Beclin-1	XM_046933362.1	F	CGTATGGCAACCACTCGTATT	97
		R	TTATTGTCCCAAGAAGAACTCAG	
<i>LC3I</i>	XM_040688401.2	F	TTACACCATATCAGATTCTTG	143
		R	ATTCCAACCTGTCCCTCA	
<i>P62</i>	XM_040682727.2	F	GACCCAGCCAAGACTACCAT	240
		R	CAGAGGCATGTAGTTTCGGC	
Beta-actin	NM_205518.2	F	TGCGTGACATCAAGGAGAAG	300
		R	TGCCAGGGTACATTGTGTA	

F = forward; R = reverse; *IL-1 β* = interleukin-1 beta; *IL-8* = interleukin-8; *TNF- α* = tumor necrosis factor-alpha; *TGF- β* = transforming growth factor-beta; *ZO-1* = zona occludens-1; *ZO-2* = zonula occludens-2; *Bax* = Bcl-2-associated X protein; *Bak* = Bcl-2 homologous antagonist/killer; *Bcl-2* = B-cell lymphoma-2.

were performed using the Prime Script RT Reagent Kit with gDNA Eraser (RR047A, Takara, Tokyo, Japan).

2.9. 16S rRNA gene amplicon sequencing

The DNA from cecal contents samples was extracted using the CTAB/SDS method. PCR amplification was conducted targeting utilizing the forward primer 338F (5'-ACTCCTACGGGAGGAGCAG-3') and the reverse primer 806R (5'-GGACTACHYGGGTWTCTAAT-3'). Library construction was performed using the NEXTFLEX rapid DNA-Seq Kit, and sequencing was conducted on the Illumina Miseq PE300 platform. The 16S rDNA sequencing was outsourced to Meiji Biomedical Technology Co., Ltd. (Shanghai, China).

The observed operational taxonomic units (OTU) were clustered based on 97% sequence similarity using the UPARSE software. Subsequently, OTU clustering analysis and taxonomic classification were performed. On this basis, alpha diversity indices, such as observed Chao1 and Shannon, were calculated, and beta diversity analysis of the bacterial community was performed using principal coordinate analysis (PCoA). To identify significantly enriched bacterial taxa (from phylum to genus) between different groups (linear discriminant analysis [LDA] score >3.0, $P < 0.05$), the linear discriminant analysis effect size (LEfSe) method was employed.

2.10. Determination of the production of short-chain fatty acids as metabolites in cecal contents

The cecal contents were weighed and added to 0.5% phosphoric acid solution (at a concentration of 10 $\mu\text{g/mL}$). The mixture was frozen, ground, and then subjected to ultrasonication for 10 min at 4 $^{\circ}\text{C}$ and centrifuged at $13,000 \times g$ for 15 min. The supernatant was collected and mixed with n-butanol for extraction. Another round of ultrasonication and centrifugation was conducted to obtain the

final supernatant, which was then analyzed using the gas chromatography-mass spectrometry (GC-MS) system (8890B-5977B gC/MSD, Agilent Technologies Inc., CA, USA). The short-chain fatty acids in the mass spectra were characterized and integrated using Masshunter Quantitative Software (version: v10.0.707.0, Agilent Technologies, CA, USA). The actual content of various short-chain fatty acids in the samples was calculated using the standard curve method.

2.11. Data analysis

All experimental data, including productivity performance, antioxidant indicators, and other variables, were subjected to statistical analysis using IBM SPSS software (version 24.0, SPSS Inc., Chicago, IL, USA). The data underwent Shapiro–Wilk and Levene's tests first. And upon meeting the assumptions, one-way analysis of variance (ANOVA) was used to test for statistical differences among different concentrations of MRY additives. If significant differences were observed among groups, Duncan's multiple comparison analysis was further used to assess pairwise differences. All data are presented as mean with standard error of the mean (SEM). $P \leq 0.05$ was considered significant, and $0.05 < P < 0.10$ was considered as a trend.

3. Results

3.1. Egg production performance and egg quality

The effects of supplementing MRY in the diet on laying performance and egg quality of laying hens are shown in Tables 3 and 4. Compared to the control group, the 0.5% and 1.0% MRY groups showed an increasing trend in egg production rate during weeks 1 to 4 ($P = 0.072$) and a significant increase during weeks 5 to 8

Table 3

Effects of marine red yeast (MRY) on egg production performance.

Item	Feeding stage	Dietary marine red yeast (MRY) additive content, %					SEM	P-value
		0 (Control)	0.5	1.0	1.5	2.0		
Daily egg production, %	wk 1–4	83.07	88.45	87.40	86.71	86.63	0.656	0.072
	wk 5–8	86.37 ^b	91.31 ^a	91.16 ^a	87.64 ^{ab}	88.68 ^{ab}	0.663	0.035
	wk 9–12	84.24	87.88	87.85	86.03	84.88	0.783	0.493
	wk 1–12	85.51	88.69	87.93	86.1	86.58	0.602	0.468
Average egg weight, g	wk 1–4	60.61	62.19	61.55	61.14	60.98	0.187	0.059
	wk 5–8	61.57	63.11	62.18	62.11	62.11	0.197	0.163
	wk 9–12	61.99	63.11	62.98	62.79	62.88	0.164	0.165
	wk 1–12	61.35 ^b	62.79 ^a	62.22 ^{ab}	62.01 ^{ab}	61.56 ^b	0.164	0.025
ADFI, kg/d per bird	wk 1–4	0.128 ^a	0.118 ^b	0.114 ^b	0.118 ^b	0.115 ^b	0.0015	0.010
	wk 5–8	0.134 ^a	0.124 ^b	0.124 ^b	0.125 ^b	0.122 ^b	0.0013	0.014
	wk 9–12	0.131 ^a	0.122 ^{ab}	0.104 ^b	0.117 ^{bc}	0.109 ^c	0.0024	0.001
	wk 1–12	0.131 ^a	0.121 ^b	0.114 ^b	0.120 ^b	0.115 ^b	0.0015	<0.001
FCR, g/g	wk 1–4	2.47 ^a	2.14 ^b	2.11 ^b	2.17 ^b	2.15 ^b	0.040	0.003
	wk 5–8	2.49 ^a	2.16 ^b	2.18 ^b	2.26 ^b	2.18 ^b	0.034	0.001
	wk 9–12	2.47 ^a	2.23 ^b	2.13 ^b	2.23 ^b	2.09 ^b	0.041	0.012
	wk 1–12	2.45 ^a	2.19 ^b	2.17 ^b	2.24 ^b	2.13 ^b	0.034	0.008

SEM = the standard error of the mean; ADFI = average daily feed intake; FCR = feed conversion ratio.

Values are presented as mean and SEM ($n = 6$).^{a-c} Within a row, means with different superscript differ at $P < 0.05$.**Table 4**

Effect of marine red yeast (MRY) additive on egg quality.

Item	Dietary marine red yeast (MRY) additive content, %					SEM	P-value
	0 (Control)	0.5	1.0	1.5	2.0		
Egg white height, mm	6.52	7.21	6.97	6.95	6.79	0.085	0.096
Yolk color	6.16 ^c	6.82 ^b	6.87 ^b	6.88 ^b	7.27 ^a	0.097	<0.001
Haugh unit	78.88 ^b	86.11 ^a	84.26 ^a	83.84 ^a	83.59 ^a	0.718	0.007
Eggshell strength, N	36.37 ^c	41.81 ^a	40.13 ^{ab}	40.32 ^{ab}	39.14 ^b	0.532	0.001
Eggshell thickness, mm	0.32	0.32	0.32	0.32	0.32	0.001	0.815
Eggshell color	29.06	28.82	29.09	28.69	29.27	0.209	0.917
Eggshell index, %	76.6 ^b	76.9	77.1	78.0	78.1	0.002	0.064

SEM = the standard error of the mean.

Values are presented as mean and SEM ($n = 10$).^{a-c} Within a row, means with different superscript differ at $P < 0.05$.

($P = 0.035$). Additionally, the average egg weight of the 0.5% MRV group showed a tendency to be higher than the control group during weeks 1 to 4 ($P = 0.059$) and was significantly higher than the control group during weeks 1 to 12 ($P = 0.025$). Moreover, the FCR of the 0.5%, 1.0%, 1.5%, and 2.0% MRV groups were significantly lower than the control group in all four feeding periods ($P = 0.003$, $P = 0.001$, $P = 0.012$, $P = 0.008$). Interestingly, except for the group supplemented with 0.5% MRV during weeks 9 to 12, the FCR of the 0.5%, 1.0%, 1.5%, and 2.0% MRV groups were significantly lower than the control group in all four feeding periods ($P = 0.010$, $P = 0.014$, $P = 0.001$, $P < 0.001$). According to Table 4, compared to the control group, the 0.5% MRV group showed a tendency of increased albumin content ($P = 0.096$), significantly increased eggshell strength ($P = 0.001$), and the 1.5% and 2.0% MRV groups showed an increasing trend in egg shape index ($P = 0.064$). The 0.5%, 1.0%, 1.5%, and 2.0% MRV groups showed significant improvements in yolk color ($P < 0.001$) and this enhancement effect was also observed in Haugh unit ($P = 0.007$), while there was no significant effect on eggshell thickness and eggshell color ($P > 0.05$). Overall, the addition of MRV additives can improve laying performance and egg quality.

3.2. Antioxidant function

Table 5 displays the effects of MRV on antioxidant indicators in laying hens during the late laying period. In the serum, compared to the control group, the 0.5%, 1.0%, 1.5%, and 2.0% MRV groups showed an increasing trend in SOD activity, while CAT activity significantly increased ($P = 0.002$). The MDA levels in the 0.5%, 1.0%, and 1.5% MRV

groups were significantly lower ($P < 0.001$). However, no significant effect was observed on GSH-Px ($P > 0.05$). In the liver, the MDA levels in the 1%, 1.5%, and 2% MRV groups were significantly lower than the control group ($P = 0.001$). There was no significant effect on CAT, SOD, and GSH-Px ($P > 0.05$). In the yolk, the 0.5%, 1.0%, 1.5%, and 2.0% MRV groups showed significantly higher SOD, GSH-Px, and CAT activities compared to the control group ($P < 0.001$, $P = 0.001$, $P = 0.002$). The MDA levels in the experimental groups were significantly lower than the control group ($P = 0.002$).

3.3. Intestinal morphology

The effects of MRV on intestinal morphology, structure, and barrier function were evaluated by observing histological sections of the intestinal microstructure, and the results are shown in Table 6. Compared to the control group, the 0.5% and 2.0% MRV groups showed a significant increase in the height of the jejunal villi ($P = 0.001$). Additionally, the C in the 0.5%, 1%, 1.5%, and 2% MRV groups significantly decreased ($P < 0.001$), while the V/C ratio significantly increased ($P < 0.001$). Moreover, compared to the control group, feeding 1.5% MRV resulted in a higher V/C ratio in the ileum of the laying hens.

3.4. Expression of genes related to intestinal barrier, autophagy and apoptosis

As shown in Fig. 1, different doses of MRV supplement did not significantly affect the tight junction protein claudin-2 in the ileum (Fig. 1A, $P > 0.05$). However, occludin (Fig. 1B, $P = 0.015$) was

Table 5

Effect of marine red yeast (MRY) additives on antioxidant indicators of laying hens.

Item	Dietary marine red yeast (MRY) additive content, %					SEM	P-value
	0 (Control)	0.5	1.0	1.5	2.0		
Serum							
SOD, U/mL	1309	1465	1447	1442	1441	19.9	0.077
GSH-Px, U/mL	1664	1664	1460	1527	1671	42.8	0.405
CAT, U/mL	0.55 ^c	1.72 ^b	2.88 ^a	2.93 ^a	2.62 ^{ab}	0.229	0.002
MDA, nmol/mL	5.66 ^a	2.81 ^{bc}	2.24 ^c	3.64 ^{bc}	4.30 ^{ab}	0.302	<0.001
Liver							
SOD, U/mg prot	868	1111	934	912	1014	43.3	0.464
GSH-Px, U/mg prot	23.74	36.95	34.11	32.45	31.12	1.533	0.440
CAT, U/mg prot	30.68	33.59	32.48	36.94	37.39	1.016	0.148
MDA, nmol/mg prot	0.59 ^a	0.54 ^a	0.42 ^b	0.45 ^b	0.44 ^b	0.020	0.001
Yolk							
SOD, U/mg prot	56.36 ^c	124.09 ^b	142.71 ^b	183.86 ^a	139.16 ^b	11.279	<0.001
GSH-Px, U/mg prot	72.90 ^b	169.63 ^a	157.69 ^a	190.85 ^a	168.70 ^a	12.115	0.001
CAT, U/mg prot	58.41 ^b	120.96 ^a	112.30 ^a	139.81 ^a	122.53 ^a	8.352	0.002
MDA, nmol/mg prot	17.23 ^a	14.00 ^b	13.71 ^b	10.53 ^c	10.27 ^c	0.768	0.002

SEM = the standard error of the mean; SOD = superoxide dismutase; GSH-Px = glutathione peroxidase; CAT = catalase; MDA = malondialdehyde.

Values are presented as mean and SEM ($n = 6$).^{a-c} Within a row, means with different superscript differ at $P < 0.05$.**Table 6**

Effects of marine red yeast (MRY) additives on intestinal morphology.

Item	Dietary marine red yeast (MRY) additive content, %					SEM	P-value
	0 (Control)	0.5	1.0	1.5	2.0		
Duodenum							
Villous height, μm	1392.27 ^a	820.37 ^c	1284.60 ^{ab}	1296.51 ^{ab}	1265.32 ^b	52.288	<0.001
Crypt depth, μm	317.03 ^a	180.52 ^b	310.97 ^a	295.86 ^a	296.46 ^a	12.753	<0.001
V/C ratio	4.65	4.87	4.19	4.05	4.2	0.189	0.604
Jejunum							
Villous height, μm	778.13 ^c	1163.27 ^a	843.74 ^{bc}	821.05 ^{bc}	985.22 ^{ab}	39.664	0.001
Crypt depth, μm	295.90 ^a	225.03 ^b	152.64 ^c	92.73 ^d	155.33 ^c	15.572	<0.001
V/C ratio	2.64 ^c	5.25 ^b	5.54 ^b	9.15 ^a	6.44 ^b	0.504	<0.001
Ileum							
Villous height, μm	590.31 ^d	632.91 ^{cd}	899.39 ^b	1097.73 ^a	722.61 ^c	42.864	<0.001
Crypt depth, μm	129.70 ^{ab}	110.29 ^b	154.97 ^a	132.31 ^{ab}	129.81 ^{ab}	4.470	0.021
V/C ratio	4.40 ^b	5.82 ^b	5.16 ^b	7.30 ^a	5.24 ^b	0.259	0.005

SEM = the standard error of the mean; V/C ratio = the villus height to crypt depth ratio.

Values are presented as mean and SEM ($n = 6$).^{a-d} Within a row, means with different superscript differ at $P < 0.05$.

significantly increased in the MRY 0.5%, 1.5%, and 2% groups. ZO-1 (Fig. 1C, $P = 0.026$) was significantly increased in the MRY 0.5%, 1%, 1.5%, and 2% groups. The tight junction protein ZO-2 showed no significant changes (Fig. 1D, $P > 0.05$). Additionally, compared to the control group, the pro-apoptotic gene *Bax* (Fig. 1E, $P < 0.001$) was significantly decreased in the MRY 0.5%, 1.0%, and 1.5% groups, while *Bak* (Fig. 1F, $P > 0.05$) showed no significant effect. The anti-apoptotic gene *Bcl-2* in the MRY 0.5% group (Fig. 1G, $P = 0.026$) was higher than in the control group. Furthermore, the expression of autophagy-related genes beclin-1 (Fig. 1H, $P = 0.002$) and *LC3I* (Fig. 1I, $P = 0.007$) was upregulated in the MRY 1.0%, 1.5%, and 2.0% groups, while the expression of *P62* (Fig. 1J, $P = 0.008$) was significantly downregulated in the MRY 0.5%, 1.0%, 1.5%, and 2.0% groups.

3.5. Serum immunoglobulins and intestinal inflammatory cytokines

Table 7 presents the changes in immune levels in the senior laying hens fed with different concentrations of MRY. The study found that when the MRY dose was 1.0%, 1.5%, and 2.0%, the concentration of IgG was significantly higher than that in the control group ($P < 0.001$). However, supplementation with MRY did not significantly increase the levels of IgA and IgM ($P > 0.05$). In terms of the expression levels of inflammatory factors in different groups, the MRY 0.5% and 1.0% groups exhibited evidently lower levels of

TNF- α , IL-1 β , and IL-8 compared to the control group ($P = 0.001$, $P < 0.001$, $P = 0.001$). Additionally, there was a trend of increased levels of the anti-inflammatory factor TGF- β with 2.0% MRY stimulation ($P = 0.083$). The qRT-PCR results in Fig. 2 show that the expression of *IL-1 β* was significantly reduced in the MRY 0.5% and 1.5% groups ($P = 0.004$). The expression of inflammatory factors *IL-8* and *TNF- α* was also significantly reduced in the MRY 0.5%, 1.0%, 1.5%, and 2.0% groups ($P < 0.001$, $P = 0.009$). However, the anti-inflammatory factor TGF- β showed higher expression in the MRY 0.5% and 1.5% groups ($P < 0.001$).

3.6. Cecal microbial diversity and community structure

We further analyzed the impact of MRY on microbial diversity by performing 16SrDNA sequencing on the cecal contents of different dose groups. As shown in the Venn diagram (Fig. 3A), the control group and the 0.5%, 1.0%, 1.5%, and 2.0% MRY groups had 247, 266, 206, 124, and 308 OTUs, respectively. Compared with the control group, there was no statistically difference between the observed species in the MRY group and the Chao1 index and Shannon index reflecting species richness and diversity ($P > 0.05$) (Fig. 3B and C). The β -diversity analysis of cecal microorganisms was based on PCoA of Bray–Curtis distance, and the flora structure of the MRY group (0.5%, 1.0%, 1.5%, 2.0%) was different from that of

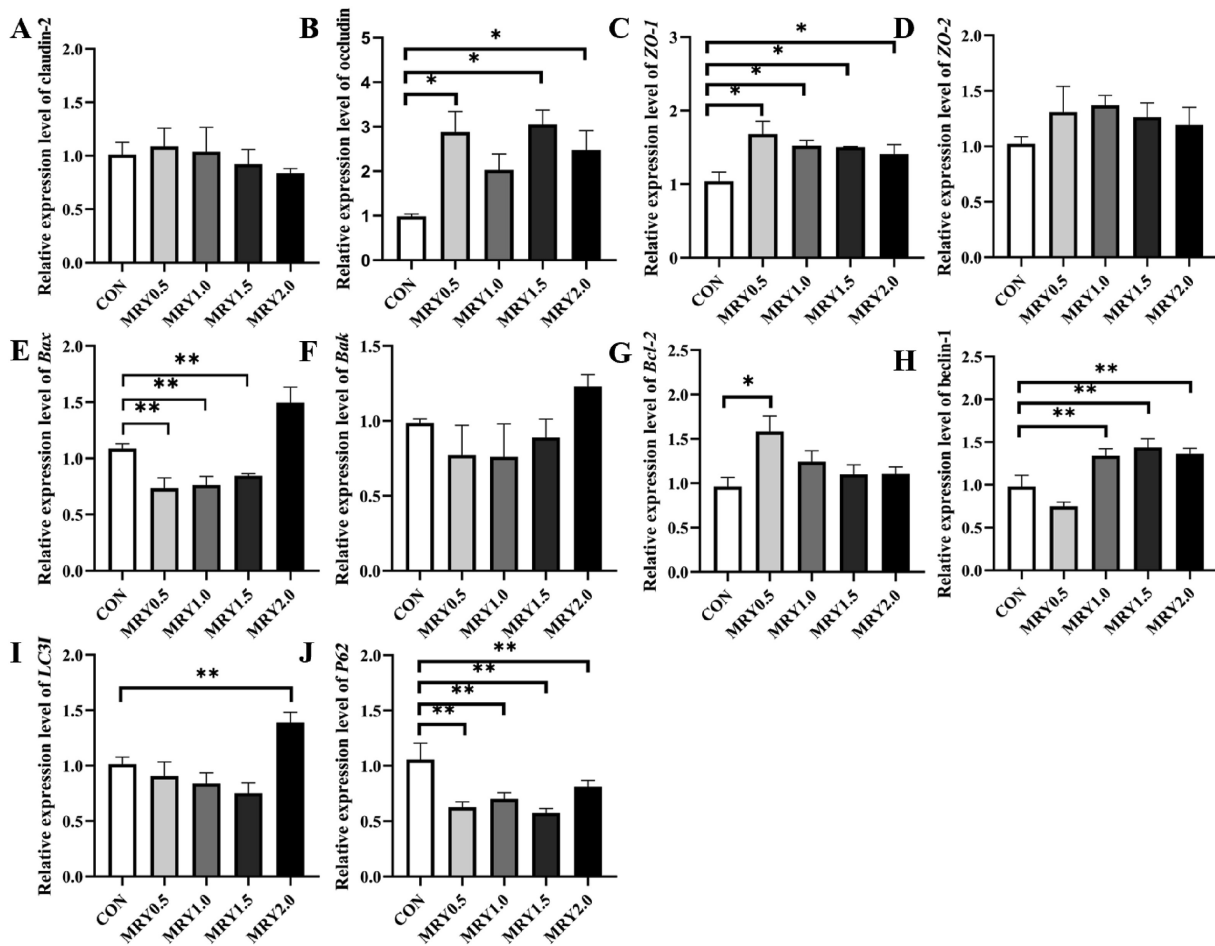


Fig. 1. The effects of marine red yeast (MRY) on the expression of intestinal barrier, autophagy, and apoptosis genes in laying hens. The mRNA expression of tight junction proteins claudin-2 (A), occludin (B), ZO-1 (C), ZO-2 (D) in the ileum. The mRNA expression of pro-apoptotic genes Bax (E) and Bak (F), and anti-apoptotic gene Bcl-2 (G) in the ileum. mRNA expression of autophagy-related proteins beclin-1 (H), LC3I (I), and P62 (J) in the ileum. The differences between the mean values $\pm$ SEM of different groups are presented in the form of a bar graph ($n = 6$). ZO-1 = zona occludens-1; ZO-2 = zonula occludens-2; Bax = Bcl-2-associated X protein; Bak = bcl-2 homologous antagonist/killer; Bcl-2 = b-cell lymphoma-2; Beclin-1 = beclin-1 autophagy-related; LC3I = microtubule-associated proteins 1A/1B light chain 3 isoform I. CON = the control group; MRY 0.5 = MRY 0.5% group; MRY 1.0 = MRY 1.0% group; MRY 1.5 = MRY 1.5% group; MRY 2.0 = MRY 2.0% group. *, $P < 0.05$; **, $P < 0.01$.

Table 7

Effect of marine red yeast (MRY) additives on immune indicators of laying hens ($\mu\text{g/mL}$).

Item	Dietary marine red yeast (MRY) additive content, %					SEM	P-value
	0 (Control)	0.5	1.0	1.5	2.0		
IgA	252.5 ^a	174.8 ^b	227.5 ^a	248.7 ^a	255.2 ^a	8.54	0.005
IgG	1618 ^b	1469 ^b	1819 ^a	1973 ^a	1822 ^a	49.7	0.001
IgM	627.0 ^a	450.8 ^c	559.1 ^{ab}	529.7 ^b	613.7 ^a	17.16	0.001
IL-1 β	3857 ^a	2688 ^c	2830 ^c	3160 ^{bc}	3555 ^{ab}	113.8	0.001
IL-8	761.6 ^a	497.4 ^c	604.4 ^{bc}	717.4 ^{ab}	694.0 ^{ab}	23.65	<0.001
TNF- α	481.6 ^a	337.1 ^b	359.6 ^b	407.3 ^{ab}	492.8 ^a	16.52	0.001
TGF- β	1573	1718	1700	1658	1911	38.2	0.083

SEM = the standard error of the mean; IgA = immunoglobulin A; IgG = immunoglobulin G; IgM = immunoglobulin M; IL-1 β = interleukin-1 beta; IL-8 = interleukin-8; TNF- α = tumor necrosis factor-alpha; TGF- β = transforming growth factor-beta.

Values are presented as mean and SEM ($n = 6$).

^{a-c} Within a row, means with different superscript differ at $P < 0.05$.

the control group (Fig. 3D, E, F, G). This suggests that MRV can positively influence the balance of the intestinal flora and affect the composition and distribution of the flora, even though it does not affect the α -diversity as a feed additive.

The cecal microbiota at the cecal phylum level mainly consisted of Bacteroidetes, Firmicutes, Actinobacteria, and Desulfobacteria (Fig. 4A). Bacteroidetes was the most abundant phylum, followed by Firmicutes, together accounting for over 80% of the total bacterial community. Compared to the relative abundance of the Firmicutes phylum in the control group at 44.53%, the 0.5%, 1.0%, 1.5%, and 2.0% MRV groups showed an increase in relative abundance, which were 50.26%, 50.82%, 49.15%, and 40.35%, respectively. However, the relative abundance of the Actinobacteria phylum in the 1% and 1.5% MRV groups, which were 36.62% and 34.41%, showed a numerical decrease compared to the control group at 37.35%. Therefore, the F/B ratio in the MRV groups was lower than that in the control group. The F/B ratio in the control group was 0.84, while the MRV groups with four different doses had values of 0.77, 0.72, 0.71, and 0.76, respectively. At the genus level (Fig. 4B), the analysis revealed that the relative abundance of the genus *Bacteroides* was greater than 20% in all four MRV experimental groups, higher than the control group's 18.29% ($P = 0.197$). Additionally, the relative abundance of *Rikenellaceae_RC9_gut_group* also showed an increase compared to the control group's 9.94%, with values exceeding 10% ($P = 0.392$). *Phascolarctobacterium* exhibited a decreasing trend ($P = 0.089$), whereas *Ruminococcus_torques_group* exhibited an increasing trend (MRY0.5%, $P = 0.096$).

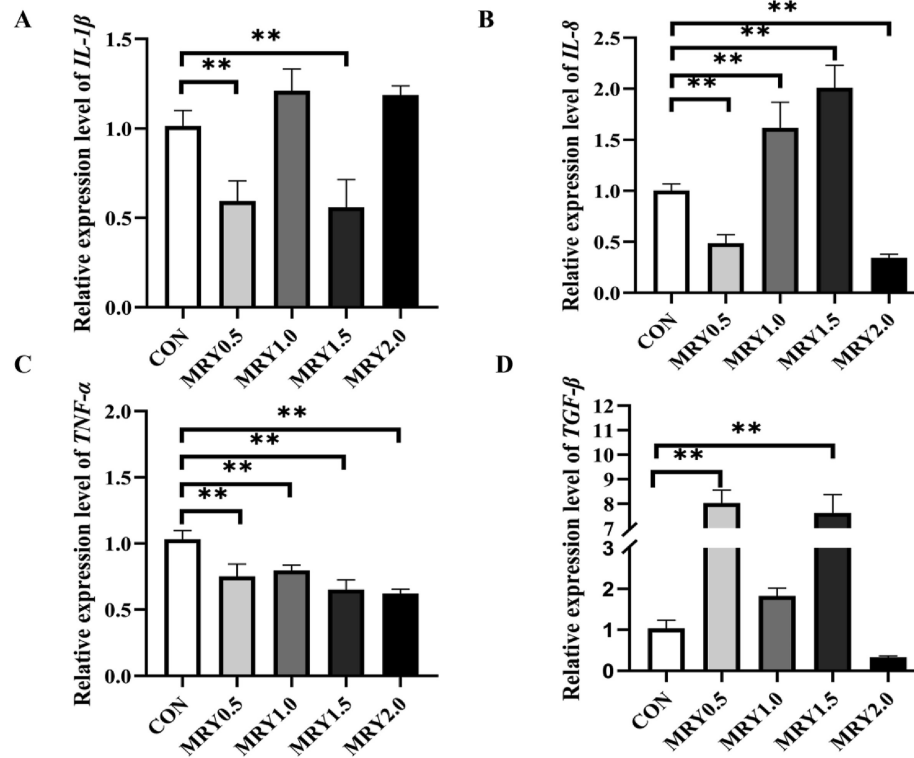


Fig. 2. The effects of marine red yeast (MRY) on serum immunoglobulin levels and intestinal inflammatory cytokines in laying hens. The mRNA expression of pro-inflammatory cytokines IL-1 β (A), IL-8 (B), TNF- α (C) in the ileum, and the mRNA expression of the anti-inflammatory cytokine TGF- β (D) in the ileum were measured. The differences between the mean values $\pm$ SEM of different groups are presented in the form of a bar graph ($n = 6$). IL-1 β = interleukin-1 beta; IL-8 = interleukin-8; TNF- α = tumor necrosis factor-alpha; TGF- β = transforming growth factor-beta. CON = the control group; MRY 0.5 = MRY 0.5% group; MRY 1.0 = MRY 1.0% group; MRY 1.5 = MRY 1.5% group; MRY 2.0 = MRY 2.0% group. **, $P < 0.01$.

LEfSe analysis was applied to identify significantly differentially abundant OTUs across the microbiota from phylum to genus (LDA score >3.0) level. The MRY group showed an overall tendency of increased beneficial bacteria compared to the control group (Fig. 4C). Specifically, the MRY 0.5% group mainly enriched *Bifidobacterium*, *Alloprevotella*, *Peptococcus*, and *Ruminococcus*; the MRY1.0% group enriched *Peptococcus* and *Ruminococcus*; the 1.5% group enriched *Sutterella*; and the MRY2.0% group significantly increased *Akkermansia muciniphila* and *Verrucomicrobi*.

3.7. Analysis of short-chain fatty acid (SCFA) metabolites in the cecum

Furthermore, we investigated the impact of MRY on the levels of SCFAs in the intestinal tract, and the results are presented in Table 8. The concentration of acetic acid in the MRY 1.5% group was significantly higher than that in the control group ($P = 0.002$). Additionally, we observed a similar trend in the MRY 1.5% group for butyric acid ($P < 0.001$), valeric acid ($P < 0.001$), and isovaleric acid ($P = 0.002$). The MRY 1.0% group also demonstrated a significant increase in the levels of propionic acid ($P < 0.001$), butyric acid ($P < 0.001$), and valeric acid ($P < 0.001$) in the intestinal tract of aged laying hens ($P < 0.05$). However, no significant changes were observed in the levels of hexanoic acid and isohexanoic acid ($P > 0.05$).

4. Discussion

Higher egg production performance and high-quality eggs are directly related to the farming profits of the enterprise. However, after the peak egg production period of high-intensity metabolism,

the egg production rate and egg quality of laying hens gradually decrease, and the average egg laying interval increases (Bar et al., 2002; Yenilmez and Atay, 2023). Our experiments found that feeding MRY can significantly increase the egg production rate, average egg weight and egg quality of laying hens, and significantly reduce FCR and average daily feed intake. The egg white height and Haugh unit are important indicators of egg freshness (Wang et al., 2019). The higher the value, the more beneficial it is to extend the shelf life of eggs. In this study, egg white height and Haugh units and yolk color were significantly increased in response to the supplementation of MRY in experimental group. Previous research has shown that supplementing yeast and yeast cell wall (YCW) can improve Haugh units (Koiyama et al., 2018; Wang et al., 2015). Adding red yeast to the diet of laying hens can enhance egg yolk color, reduce serum and yolk cholesterol levels, and increase duodenal villus height (Tapingkae et al., 2016; Tapingkae et al., 2018), which is consistent with our present study. It has been reported that dietary supplementation with *Bacillus coagulans* X26 can improve the egg production performance and egg quality of laying hens in the late laying period by improving intestinal health (Xu et al., 2022). Therefore, we speculate that the beneficial effect of MRY on egg production performance may also be exerted through the intestine.

Under normal physiological conditions, there exists a dynamic equilibrium between free radicals and antioxidants in the body (Poprac et al., 2017). However, during the late laying period, excessive ovulation in laying hens leads to the continuous accumulation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), resulting in an imbalance of oxidative stress. This imbalance critically damages reproductive performance, causes tissue damage, and accelerates aging (Surai et al., 2019; Pisoschi

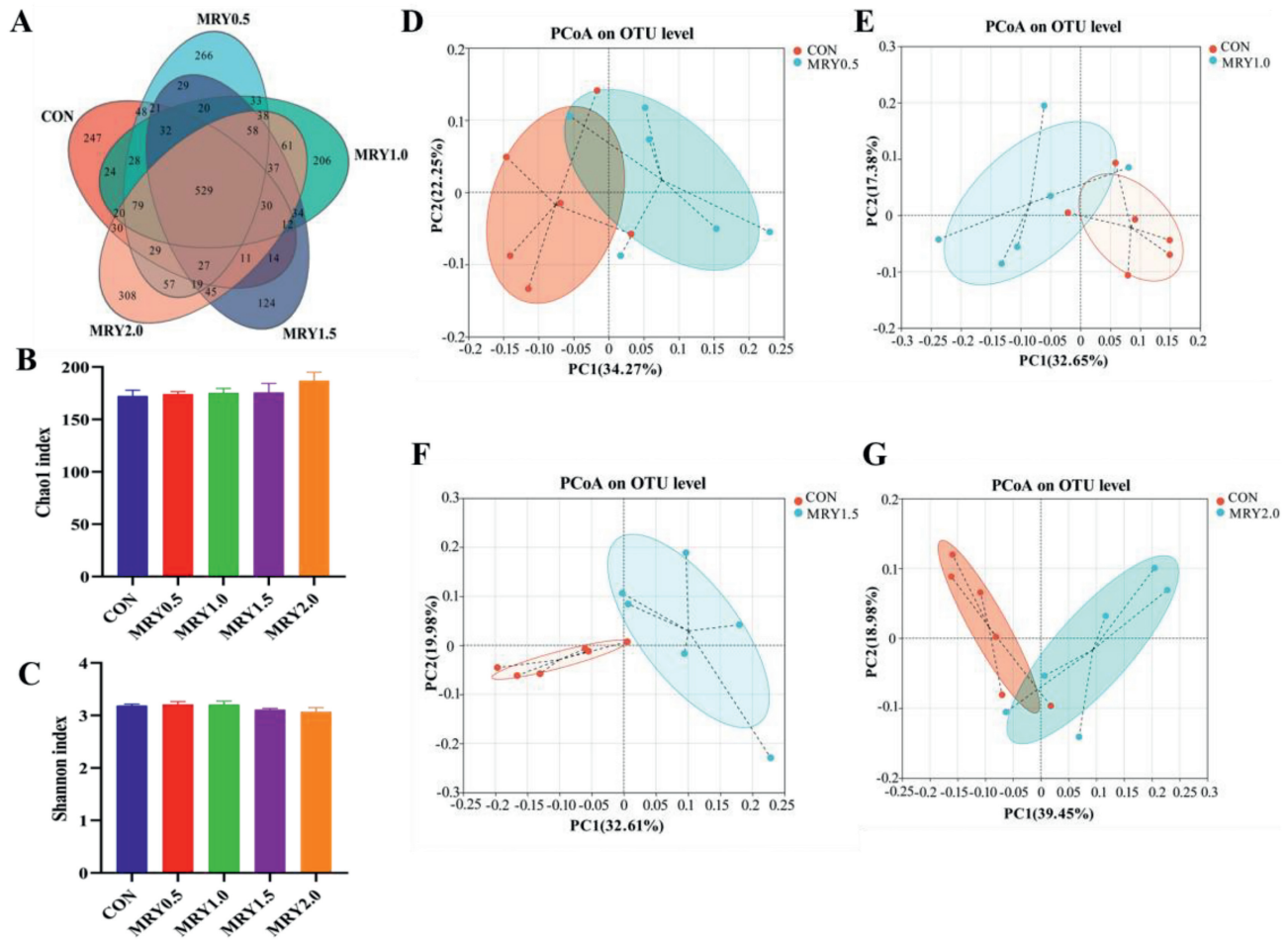


Fig. 3. The impact of marine red yeast (MRY) on the microbial species composition and diversity in laying hens. (A) Species Venn plot. (B and C) Alpha-diversity: Chao1 and Shannon indexes at OTU level. (D to G) Beta-diversity: Principal coordinate analysis (PCoA) of MR0.5%, 1.0%, 1.5%, 2.0% groups; CON = the control group; MR0.5 = MR0.5% group; MR1.0 = MR1.0% group; MR1.5 = MR1.5% group; MR2.0 = MR2.0% group.

et al., 2021). It can be inferred that oxidative stress is closely related to the decline in egg production performance in laying hens. Deng et al., (2012) found that probiotics can counteract the adverse effects of oxidative stress on laying hens by increasing the concentrations of SOD and other antioxidants. Moreover, this protective effect is particularly prominent during the late stage of the egg-laying period (Deng et al., 2012; Xu et al., 2023). In our study, the addition of MRY significantly elevated the levels of CAT, GSH-Px and SOD activity in laying hens compared to the control group, while significantly reducing MDA levels. These findings are consistent with research on other probiotics, such as *Clostridium butyricum* (Zhan et al., 2019). It has been reported that yeast wall polysaccharides can exert free radical scavenging effects by binding to cell-specific surface molecules (Ben Farhat et al., 2015; Du et al., 2015; Yuan et al., 2010). Moreover, supplementation with *Lactobacillus plantarum* 16 (Lac16) can regulate intestinal barrier function by increasing intestinal CAT levels (Wu et al., 2019). This evidence shows that MRY can effectively scavenge oxidative free radicals in aging laying hens by stimulating antioxidant enzyme components and resist oxidative damage to organs such as intestines, thereby improving egg production rate and egg quality.

The digestion and absorption process of nutrients mainly occurs in the small intestine. The small intestine's villi, which extend into the intestinal lumen, serve as the primary barrier for nutrient digestion and absorption. The development and health of the

animal's intestines are typically assessed based on three indicators: villus height, crypt depth, and V/C ratio (Lei et al., 2015). Our results unequivocally demonstrate that supplementation with MRY leads to higher villus height and V/C ratio in the jejunum, shallower crypt depth, and increased villus height in the ileum. These findings align with previous studies indicating that supplementation of *Sporidiobolus pararoseus* in laying hen diets and *Saccharomyces cerevisiae* in broiler diets improves intestinal health (Muthusamy et al., 2011; Tapingkae et al., 2018). Other relevant research has reported that supplementing YCW in laying hen diets enhances the length and V/C ratio of small intestinal villi (Kyoung et al., 2023). This may be due to certain components of YCW, such as β -glucan and MOS, which protect the mucosa by preventing contact between villi and pathogens or disease-causing bacteria. Furthermore, YCW can increase the number of goblet cells, stimulate epithelial cell renewal, and enhance mucin secretion (Gao et al., 2008; Muthusamy et al., 2011; Spring et al., 2000). Overall, supplementation with MRY significantly reduced intestinal villus damage and enhanced intestinal morphological parameters like villus height and V/C, thereby improving the structural integrity of the intestinal epithelium and promoting nutrient absorption, which may further contribute to the increased FCR.

Previous reports have indicated that during the aging process, there is an increase in intestinal permeability ("leaky gut") (Tremblay et al., 2017; Schmitz et al., 1999), and the integrity of the

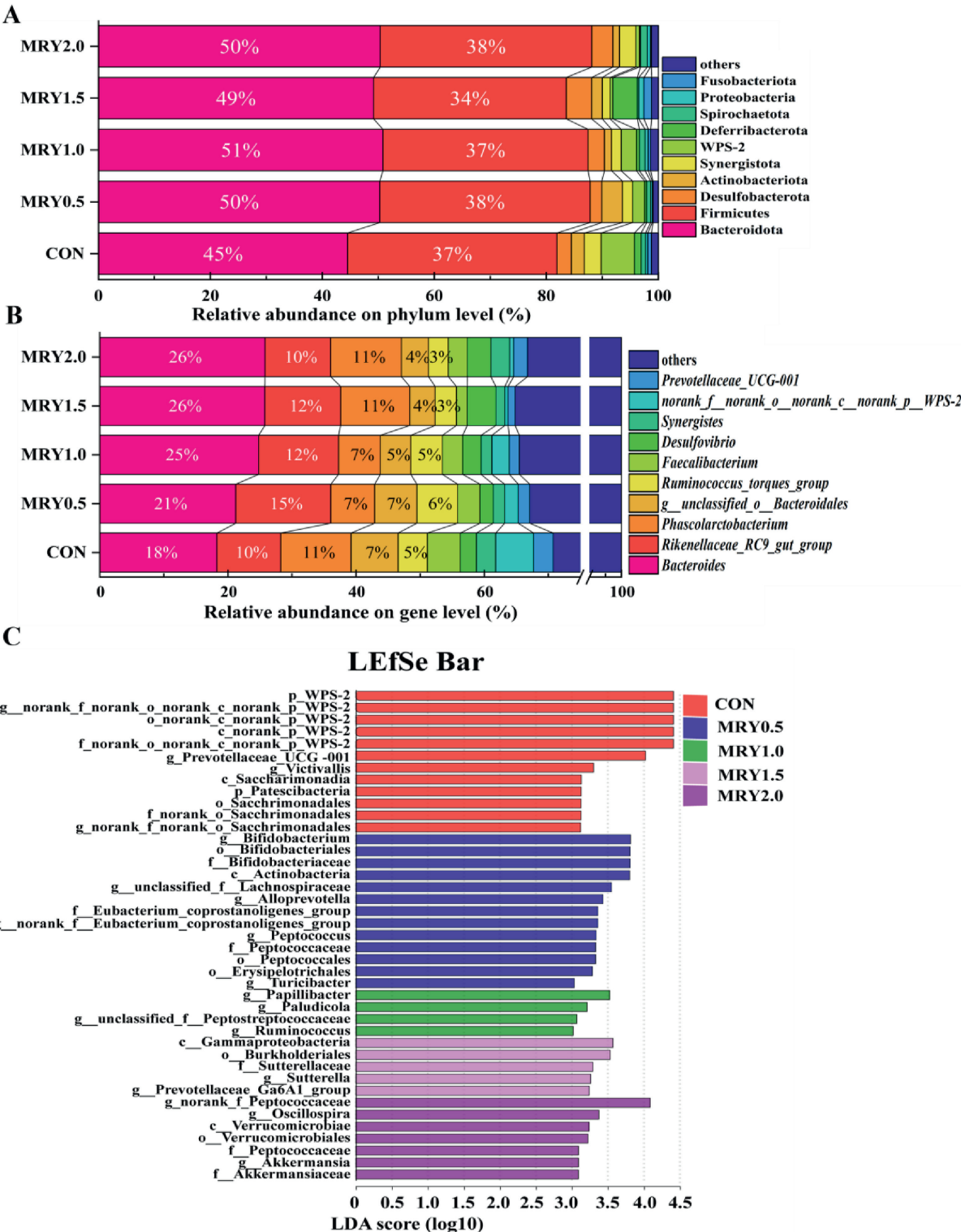


Fig. 4. The impact of marine red yeast (MRY) on the relative abundance of microbial communities and enriched bacterial taxa in laying hens. Relative abundance of microbiota at phylum level (A) and genus level (B), and (C) linear discriminant analysis of gut microbiota effects (LDA score >3.0, $P < 0.05$). CON = the control group; MRY 0.5 = MRY 0.5% group; MRY 1.0 = MRY 1.0% group; MRY 1.5 = MRY 1.5% group; MRY 2.0 = MRY 2.0% group.

intestinal barrier is compromised. Tight junction proteins play a crucial role in maintaining intercellular permeability and functioning as a barrier. They can selectively screen out molecules and microorganisms in the intestinal cavity that are beneficial to the body and gradually regulate their absorption (Schmitz et al., 1999).

Studies have shown that the expression levels of tight junction proteins decrease with age (Tremblay et al., 2017). Our results showed that MRY could promote the expression of ileal tight junction proteins occludin and ZO-1. Therefore, we hypothesize that the upregulation of tight junction proteins promotes the

Table 8Effect of marine red yeast (MRY) additives on intestinal short-chain fatty acids of laying hens ($\mu\text{g/g}$).

Item	Dietary marine red yeast (MRY) additive content, %					SEM	P-value
	0 (Control)	0.5	1.0	1.5	2.0		
Acetic acid	2108 ^b	2982 ^{ab}	2831 ^{ab}	3748 ^a	2092 ^b	166.3	0.002
Propionic acid	782 ^b	1045 ^{ab}	2667 ^a	1427 ^{ab}	916 ^{ab}	149.3	<0.001
Butyric acid	349 ^b	49 ^{b0}	766 ^a	792 ^a	442 ^b	44.3	<0.001
Isobutyric acid	61 ^b	94.6 ^b	75.2 ^b	195.4 ^a	62.2 ^b	11.90	<0.001
Isovaleric acid	129.5 ^b	132 ^b	156.8 ^b	248.4 ^a	127.3 ^b	11.84	0.002
Valeric acid	36.3 ^c	76.5 ^b	160.8 ^a	174.5 ^a	51.5 ^c	15.24	<0.001
Isohexanoic acid	5.60	5.80	6.20	6.20	6.30	0.130	0.430
Hexanoic acid	4.70	4.80	4.80	5.30	4.60	0.110	0.457

SEM = the standard error of the mean.

Values are presented as mean and SEM ($n = 6$).^{a-c} Within a row, means with different superscript differ at $P < 0.05$.

integrity of the intestinal barrier in aged laying hens and accelerates the repair of damage. This hypothesis is supported by the experimental evidence presented by Zhang et al., (2020).

Autophagy is an important lysosomal catabolic mechanism involved in the degradation of damaged and aging cellular proteins and organelles (Yadav et al., 2016). There exists a complex inter-relationship between intestinal function and autophagy, and dysfunctional autophagy can trigger intestinal inflammation, accumulation of ROS, and contribute to the development of intestinal diseases such as inflammatory bowel disease (IBD) and colorectal cancer (Burada et al., 2015; Pott et al., 2018). Studies have shown that autophagy proteins can be used as new biomarkers of age-related diseases (Miceli et al., 2023), and the abundance of autophagy-related proteins gradually decreases with age. Our results show that supplementation of MRY significantly upregulates autophagy-related genes, namely *LC3I* and *beclin-1*, while down-regulating the expression of *P62*. These changes enhance intestinal autophagy activity, thereby delaying intestinal aging and maintaining intestinal health. Furthermore, the loss of the tight junction protein occludin results in increased intestinal permeability and cell apoptosis. However, overexpression of the intestinal anti-apoptotic gene *Bcl-2* can prevent and alleviate intestinal barrier dysfunction (Beeman et al., 2012; Otani et al., 2020). From the results, it is clear that there was a significant decrease in the pro-apoptotic genes *Bax* and *Bak*, along with a significant increase in the anti-apoptotic gene *Bcl-2* in the experimental group. This indicates that MRY can reduce intestinal epithelial cell apoptosis and improve intestinal permeability through tight junctions. Considering the aforementioned research results, MRY enhances the absorption surface area through improved villus structure, increased levels of tight junction proteins and enhanced autophagic apoptosis activity. This maintenance of normal renewal of intestinal epithelial cells and alleviation of age-related intestinal diseases contribute to the prevention of intestinal damage, improvement in intestinal permeability, and promotion of intestinal health.

Increased inflammatory status is another hallmark of intestinal aging (Thevaranjan et al., 2017). As the body gradually ages, there is a significant increase in the expression levels of inflammatory cytokines such as *IL-1 β* derived from macrophages, while the expression levels of anti-inflammatory cytokines are relatively lower. This imbalanced state directly affects intestinal permeability (Maij  et al., 2014; Thevaranjan et al., 2017). It has been observed that intestinal inflammation in laying hens can result in impaired nutrient absorption and the entry of endotoxins into the liver, causing liver inflammation that inhibits the synthesis of yolk precursors. Consequently, this disrupts normal follicle growth and leads to a decrease in egg production (Nii et al., 2020). Adding probiotics and probiotic complexes to the diet has been shown to enhance the serum immunoglobulin concentration in chickens and

exert inhibitory effects on the expression of inflammatory cytokines (Qiu et al., 2021; Wang et al., 2021; Zhou et al., 2020). We observed that the serum levels of immunoglobulins, such as IgG, in normal aging laying hens were low. However, supplementation with MRY, as a functional probiotic, significantly increased serum IgG levels and the intestinal anti-inflammatory factor TGF-1 β content when added to the feed. Moreover, the expression of intestinal pro-inflammatory factors *IL-1 β* , *IL-8*, and *TNF- α* was significantly reduced, which is similar to the finding that *Bacillus licheniformis* can enhance the egg production performance of laying hens by regulating immune function (Wang et al., 2017). Based on these observations, we speculate that MRY additives as a dietary supplement for laying hens can improve the imbalance among intestinal inflammatory factors in aging laying hens by stimulating the local immune system and systemic immune factors in the intestines. This, in turn, exerts an immunomodulatory effect, and promotes nutrient absorption, and increases egg production.

The gut microbiota serves as a crucial regulator of host homeostasis (Gerritsen et al., 2011). It has been confirmed that gut dysbiosis is significantly associated with accelerated aging and chronic inflammation, and it leads to impaired intestinal barrier function (Bodogai et al., 2018; Hall et al., 2005; Sugihara et al., 2022). Recent studies have demonstrated alterations in bacterial diversity within the intestinal microbiome during intestinal aging, characterized by a decline in beneficial microorganisms and an increase in potential pathogenic bacteria (Agus et al., 2021; Biagi et al., 2010; Mariat et al., 2009.). Notably, the experimental group exhibited an increasing trend in the abundance of Bacteroidetes, the Firmicutes to Bacteroidetes ratio (F/B) and *Rikenellaceae_RC9_gut_group*. Bacteroidetes have the capacity to hydrolyze indigestible polysaccharides, generating SCFAs (Liu et al., 2021). Decreased F/B ratio can enhance host energy metabolism (Li et al., 2020), while *Rikenellaceae_RC9_gut_group* promotes carbohydrate digestion and absorption in the intestine (Berry et al., 2015). In this experimental study, we observed that after the addition of MRY, the abundance of obligate anaerobes such as *Bifidobacterium*, *Alloprevotella*, *Peptococcus*, *Ruminococcus* and other bacteria that decompose oligosaccharides and produce SCFAs in the intestine to regulate intestinal homeostasis increased. At the same time, the content of SCFAs such as acetic acid, propionic acid also increased significantly. Short-chain fatty acids, the primary metabolites of polysaccharides fermented by microorganisms, affect a variety of host activities, such as intestinal pH regulation, energy utilization, and resistance to intestinal inflammation (Miao et al., 2020). Acetic acid and propionic acid possess the ability to inhibit the release of pro-inflammatory cytokines from macrophages, thus acting as potential anti-inflammatory mediators (Zafar and Saier, 2021). Butyrate is an energy source for intestinal epithelial cells and exerts beneficial effects on intestinal development and intestinal

epithelial cell integrity (Liao et al., 2020). Short-chain fatty acids not only facilitate the absorption of nutrients by improving the intestinal structure but also regulate systemic calcium homeostasis by influencing calcium absorption, deposition, and solubility through pH modulation of the host intestinal lumen, thereby enhancing egg quality (Feng et al., 2021; Gultemirian et al., 2014; Montalvany-Antonucci et al., 2019; Zaiss et al., 2019). Of note was the significant increase in *Sutterella* and *A. muciniphila* in the experimental group. *Sutterella* is a potential probiotic that helps improve the feed conversion efficiency of chickens; while insufficient *Sutterella* may lead to inflammatory bowel disease, which is associated with a weakened ability of the intestines to resist harmful substances (Biasato et al., 2019; Xie et al., 2020). *A. muciniphila* may be a potential therapeutic target for aging and aging-related metabolic disorders by regulating host immunity and restoring gut microbiome homeostasis mediated by increased *A. muciniphila* abundance and its metabolites, such as butyrate (Jian et al., 2023; Shin et al., 2021; Wang et al., 2020). In summary, the inclusion of MRY in the diet increases the relative abundance of beneficial bacteria and the concentration of SCFAs. These microbial communities and metabolites have shown significant effects in maintaining intestinal morphology, modulating immune responses, and promoting nutrient absorption and utilization. Therefore, it can be concluded that they contribute to the promotion of intestinal health and production performance in aged laying hens.

5. Conclusion

In summary, this study demonstrates that MRY supplementation enhances the body's immunity, antioxidant capacity, intestinal autophagy, apoptosis, and induces changes in intestinal microbiota composition and metabolites. These effects alleviate the state of intestinal aging in laying hens, restoring intestinal homeostasis and improving the digestion and utilization of nutrients in feed. Consequently, it positively impacts the production performance and egg quality of laying hens. Notably, there was no significant difference observed between high and low doses of MRY additives in terms of their effects on the production performance and intestinal health during the late laying period. Therefore, for the sake of production economic benefits, it is recommended to use a low dose of 0.5% in the diet of laying hens. These results provide important evidence for MRY as a novel poultry feed additive that could prolong the peak egg production period of laying hens by restoring intestinal homeostasis. Further verification at the cellular level could be considered in future studies.

Author contributions

Yudian Zhao: Investigation, Formal analysis, Visualization, Data management and Writing – Original draft. **Sujin Si:** Methodology, Data organization, Validation, supervision, Writing – Original draft. **Yangguang Ren, Xing Wu, Jing Li, Xueyang Yao and Zhaozheng Xu:** Writing – Reviewing and Editing. **Zihao Zhang, Yixiang Tian, Yijie Li and Meng Hou:** Assistance in Sample collection and Data collection. **Ruirui Jiang, Xiangtao Kang and Yujie Gong:** Investigation and Conception. **Qiang Li:** Investigation and Experiment design. **Yadong Tian:** Conceptualization, Writing – Reviewing and Editing, Funding acquisition and Project administration.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal

interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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