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Original Research Article

Compound *Bacillus* improves eggshell quality and egg metabolites of hens by promoting the metabolism balance of calcium and phosphorus and uterine cell proliferation

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ABSTRACT

Probiotics have beneficial effects on improving egg quality, but there is little research about the effect of probiotics on metabolite composition, and the mechanisms are not yet fully understood. The aim of this study was to investigate the potential mechanisms by which compound *Bacillus* improves egg quality and metabolite composition. A total of 20,000 Jingfen No. 6 laying hens at 381 d old were randomly divided into two treatments: control group with a basal diet, and the basal diet with 5×10^8 CFU/kg compound *Bacillus* supplementation (Ba) group. The trial lasted eight weeks. The results showed that compound *Bacillus* improved the gloss and strength of eggshells and reduced the ratio of sand-shell eggs by 23.8%. Specifically, the effective layer of eggshell was thicker and its calcite column was closely connected. Compound *Bacillus* increased the contents of beneficial fatty acids in the egg yolk, and lipids and lipid-like molecules in the albumen ($P < 0.01$), while decreased the contents of total cholesterol, triglycerides, and benzene ring compounds in the egg yolk and organic oxygen compounds in the albumen ($P < 0.01$). In addition, the compound *Bacillus* increased the calcium absorption in the duodenum by up-regulating the expression of transporters and serum hormone synergism ($P < 0.05$), and promoted metabolic balance of calcium and phosphorus. Simultaneously, uterine transcriptome showed that the expression of ChaC glutathione specific gamma-glutamylcyclotransferase 1 (*CHAC1*), glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1 (*C1GALT1*), phosphatidylinositol-4-phosphate 5-kinase type 1 beta (*PIP5K1B*), methylenetetrahydrofolate dehydrogenase 2 (*MTHFD2*), brain enriched myelin associated protein 1 (*BCAS1*), and squalene epoxidase (*SQLE*) genes were increased ($P < 0.01$), indicating that nutrient metabolism activity was enhanced. The expression of the *BCAS1, C1GALT1,* KLF transcription factor 13 (*KLF13*), and leucine rich repeat neuronal 1 (*LRRN1*) was increased ($P < 0.01$), indicating that the cell proliferation was enhanced, which slowed uterus aging. In conclusion, compound *Bacillus* improved the eggshell strength and metabolite composition in the egg by promoting metabolic balance of calcium and phosphorus, cell proliferation, and nutrient metabolism in the uterus.

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1. Introduction

In recent years, biological feed additives have been widely used in poultry, including plant extracts, essential oils, essential fatty acids, probiotics, prebiotics, exogenous enzymes, and acidifying additives (Marimuthu et al., 2022; Varmuzova et al., 2015). They can improve the growth performance and productivity of animals by increasing intestinal health, digestibility, feed utilization (Saleh et al., 2021), immunity (Kholif et al., 2021), anti-inflammatory response, antioxidant capacity, and pro-mitochondria biogenesis (Basharat et al., 2023; Dai et al., 2022; Mendonça et al., 2021). Previous studies have shown that probiotics have the potential to increase the average weight of eggs, improve shell quality, and reduce the number of broken and soft-shelled eggs (Nishiyama et al., 2021; Xiang et al., 2019) by influencing the structure of the intestinal microbiota (Khogali et al., 2022), including reducing the colonization of pathogens (Khan and Chousalkar, 2020) and increasing the production of beneficial metabolites such as short-chain fatty acids (Melara et al., 2022). The uterus is a crucial organ of egg formation where the shell membrane of eggs forms until eggshell mineralization (Qi and Wang, 2023). Probiotics could improve eggshell quality by promoting the metabolic balance between calcium and phosphorus (Nishiyama et al., 2021; Zou et al., 2021), affecting amino acid, carbohydrate, and lipid metabolism (Zhu et al., 2022), and the immune function of oviduct mucosa (Nii, 2022). However, the mechanism by which probiotics affect the metabolite composition of eggs by regulating uterine gene expression remains elusive.

Previous studies in our lab indicated that *Bacillus amyloliquefaciens* SC06 (BaSC06) could promote the activation of M2 macrophages, enhance their bactericidal ability to reduce the abundance of harmful microorganisms, and enhance barrier function in the intestine, thus improving intestinal inflammation (Cao et al., 2020; Fu et al., 2019; Wang et al., 2018). *Bacillus subtilis* B10 (B10) could improve the intestinal barrier by enhancing intestinal tight junction proteins and increasing the concentration of immunoglobulin A (IgA) in the intestine, activating the Toll-like receptor signaling pathways, and thereby enhancing the immune function of the intestinal mucosa (Rajput et al., 2017, 2013; Xu et al., 2022a,b). In addition, we found that BaSC06 could improve egg production and quality, and that compound *Bacillus* of B10 and BaSC06 exerted a positive impact on the systemic health, production performance, and egg quality of laying hens, especially eggshell quality (Xu et al., 2022a,b). However, the deeper impact and mechanism of compound *Bacillus* on egg quality are not yet fully understood. Therefore, we evaluated the effects of compound *Bacillus* on the quality and nutritional composition of egg products and explored its mechanism by using metabolomics, transcriptomics, and RT-qPCR.

2. Materials and methods

2.1. Animal ethics statement

All experimental procedures were conducted following the Animal Welfare Committee guidelines and the experimental protocol was approved (permission number: ZJU20160416) by the Animal Care and Use Committee of Zhejiang University (Hangzhou, China). All sections of this report adhere to the ARRIVE Guidelines for reporting animal research (Kilkenny et al., 2012).

2.2. Probiotic preparation

Compound *Bacillus* (including BaSC06 and B10, both isolated and preserved in our laboratory) was prepared by our laboratory as follows. BaSC06 and B10 were separately cultured in Luria–Bertani (LB) broth at 37 °C overnight under aerobic conditions, and then BaSC06 and B10 pellets were collected after centrifugation at 3500 × *g* for 10 min at 4 °C. Then, the pellets were washed three times with sterile phosphate-buffered saline (PBS, pH 7.2). Finally, the pellets were diluted by starch and the two *Bacillus* were mixed at a ratio of 1:1 to form compound *Bacillus* which contained *Bacillus* at 1×10^9 colony-forming units (CFU)/g.

2.3. Experimental design

The experiment was performed at Tiansheng Ecological Agriculture Co., Ltd., Zhejiang, China. In this experiment, a total of 20,000 Jingfen No. 6 laying hens at 381 d old in the late laying stage with similar performance were randomly allotted to two dietary treatment groups, with three replicates for each group. The control (Con) group were fed a basal diet. The other group were fed the basal diet supplemented with 5×10^8 CFU/kg compound *Bacillus* (Ba). The compound *Bacillus* were mixed with premixes at first and then with the other ingredients to make a high uniformity of the feed mixing. The laying hens were housed in staggered three-layer cages, with each cage accommodating 15 hens. The cages (0.44 m long × 0.30 m wide × 0.45 m high) were equipped with nipple drinkers and trough feeders. The hens were reared according to the breed standards. The light program consisted of 16 h light per day and the average temperature was maintained at 26 ± 3 °C during the entire experimental period (eight weeks). The first week was an adaptation period, and the following seven weeks were the formal experiment. During the preliminary experiment, the laying rate of laying hens was observed and two groups were adjusted, so that there was no statistical difference for laying rate between two groups. During the entire experimental period, hens were free to drink and were fed twice a day at 07:30 and 15:00. Immunization was carried out according to the routine immunization program.

2.4. Experimental diet

The feed formula for laying hens was based on a corn-soybean basal diet, with reference to the recommended nutritional levels for laying hens in NRC (1994). The composition and nutritional level of the basal diet used in the experiment are shown in Table 1. Metabolizable energy was calculated by referring to Table of China Feed Composition and Nutritional Values (Institute of Animal Science of CAAS and other institutes, 2022). Crude protein, calcium, total phosphorus, amino acids and sulfur amino acids were determined according to China national standards (GB/T 6432-2018), (GB/T 6436-2002), (GB/T 6437-2018), and (GB/T 18246-2019), and (GB/T 15399-2018), respectively.

2.5. Egg sampling procedure

2.5.1. Sample collection and egg quality measurements

At the end of the 8-week experiment period, 40 eggs were randomly selected from each group, with eight eggs from each replicate, for the analysis of egg quality and calculating the percentages of sand-shell eggs. Then, the gloss on the egg equator was evaluated according to the specific method in Table 2.

Table 1
Ingredients and nutrient levels of basal diets (air-dry basis, %).

Item	Content
Ingredients	
Corn	57.00
Soybean meal, 46% CP	24.00
Wheat middling	5.50
Soybean oil	1.00
Limestone	9.00
Dicalcium phosphate	1.00
Salt	0.30
DL-Methionine	0.12
Lysine-HCl	0.08
Premix[1]	2.00
Total	100.00
Nutrient level[2]	
Metabolizable energy, Mcal/kg	2.95
Crude protein	16.43
Lysine	0.89
Methionine	0.40
Threonine	0.64
Tryptophan	0.22
Cysteine + Methionine	0.75
Calcium	3.62
Total phosphorus	0.56
Available phosphorus	0.35

[1] Provided the following per kilogram of premix: vitamin A, 6250 IU; vitamin D_3, 3125 IU; vitamin E, 15 IU; vitamin K_3, 2 mg; vitamin B_1, 1 mg; vitamin B_2, 8.5 mg; vitamin B_5, 50 mg; vitamin B_3, 32.5 mg; vitamin B_6, 8 mg; folic acid, 5 mg; vitamin B_{12}, 5 mg; vitamin H, 1 mg; choline chloride, 500 mg; I, 0.35 mg; Fe, 60 mg; Mn, 65 mg; Zn, 66 mg, and Se, 0.3 mg.
[2] Metabolizable energy was calculated and the others were measured values.

The 40 eggs were divided into two groups at random. Half of the eggs were used to test the egg quality within 24 h, and the other half were weighed and then placed at room temperature (about 30 °C) for 30 d in order to evaluate the preservation effect of compound *Bacillus* on eggs by testing the egg quality. Egg weight, eggshell breaking strength, protein height, Haugh unit, and yolk color were measured with an egg quality analyzer (DET-6000, Japan). Eggshell color was visually estimated using an eggshell color guide (Zinpro Corp, P-7060). Eggshell thickness was measured on the egg equator at 3 points by a micrometer screw (0.001-mm micrometer IP 54, Wilson Wolpert, Maastricht, the Netherlands) and the average eggshell thickness was calculated for every egg. The yolk weight and eggshell weight were weighed. The yolk ratio and eggshell ratio were calculated. The length and width of egg were measured with vernier calipers, and then the egg shape index calculated. Yolk ratio, eggshell ratio, and egg shape index were calculated as follows:

$$\text{Yolk ratio } (\%) = \frac{\text{yolk weight (g)}}{\text{egg weight (g)}} \times 100,$$

$$\text{Eggshell ratio } (\%) = \frac{\text{eggshell weight (g)}}{\text{egg weight (g)}} \times 100,$$

$$\text{Egg shape index} = \frac{\text{egg length (mm)}}{\text{egg width (mm)}},$$

Loss of egg weight = average egg weight within 24 h − average egg weight after 30 d.

2.5.2. Eggshell ultrastructural assessment

Two eggs were randomly selected from each group, and two small pieces of fragments (about 0.25 cm^2) from the equator of each egg were mounted on a stub to expose the outer surface and cross section. The sample was coated with gold-palladium in a ion sputter (Hitachi Model E-1010, Japan) for 4 to 5 min, then the outer surface was observed through a scanning electron microscope (Hitachi SU-8010, Japan) at a standard magnification of 300× and the cross section with standard magnifications of 200× and 4000×.

2.5.3. Egg chemical composition

The fatty acid composition of the eggs was determined from six eggs randomly selected in each treatment, and the total cholesterol (TCHO; catalog no. A111-2-1) and total triglycerides (TG; catalog no. A110-2-1) measured in the yolks of two groups of eggs according to the manufacturer's instructions of the kit from Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China.

2.5.4. Metabolomics of the yolk and albumen

Six eggs were selected as mentioned in 2.5.3 for the metabolomics. In this project, a high-resolution mass spectrometer (Q Exactive, Thermo Fisher Scientific, USA) was used to conduct non-targeted metabolomics detection on 12 eggs samples by using liquid chromatograph mass spectrometer/mass spectrometer technology to collect data of positive ion (pos) and negative ion (neg) modes respectively, to explore the metabolomics composition and biological function of the samples.

2.6. Tissue sampling procedure

2.6.1. Sample preparation

At the end of the 8-week experiment, 15 laying hens from each treatment group randomly were selected and slaughtered, with three hens from each replicate. These laying hens were deprived of

Table 2
The standard of gloss state evaluation in eggs.

Gloss level	Judging standard
3	The texture of the eggshell surface is exquisite. The coating is evenly distributed over the eggshell. The ability of reflecting light is strong, and obvious light reflection points can be seen.
2	The texture of the eggshell surface is relatively smooth. The coating is distributed over most of the eggshell surface, but the thickness is uneven. The ability of reflecting light is mediocre, and the light reflection points can be seen.
1	The texture of eggshell surface is slightly rough. The coating is thin and the coverage is small. The ability of reflecting light is weak, and is mainly diffuse reflection, and the light reflection point cannot be seen.
0	The texture of eggshell surface is rough and has particles. Almost no coating. The reflecting light cannot be seen and is mainly diffuse reflection.

feed for 12 h, but water was offered ad libitum. Then blood from the carotid artery were sampled using vacuum blood tubes. The samples were centrifuged for 10 min (3000 × *g*) to separate the serum then stored in Eppendorf tubes at − 80 °C. In addition, laying hens were euthanized for collection of tissues. The whole duodenum, uterus, and intact kidney of laying hens were sampled, snapped frozen in liquid nitrogen, and then stored at −80 °C for further RNA extraction and RT-qPCR analysis.

2.6.2. Serum biochemistry

The serum biochemistry was determined from 12 hens randomly selected in each treatment. The separated serum was used to measure the contents of 1, 25−dihydroxy vitamin D_3 (DHVD3; catalog no. H191-2), 25-hydroxy vitamin D_3 (25HVD3; catalog no. H191-1), calcitonin (CT; catalog no. H153), parathyroid hormone (PTH; catalog no. H207), and thyroid hormone (T4; catalog no. H223) by using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China). Briefly, serum samples were added to enzyme wells, which had been pre-coated with antibodies specific to the antigen recognition. Horse radish peroxidase-labelled antigen was then added. Following incubating for 30 min at 37 °C, horse radish peroxidase competed with solid phase antigen and formed immune complex. Then the combined horse radish peroxidase catalyzed tetramethy1 benzidine into blue, which then turned yellow due to the action of acid. The absorbance of each well was determined under 450 nm wavelength using a microplate reader (SpectraMax M5, Molecular Devices, San Jose, CA, USA). Concentrations of serum calcium (Ca; catalog no. C004-2-1) and inorganic phosphorus (P; catalog no. C006-1-1) were determined according to the kit manufacturer's instructions (Jiancheng Bioengineering Institute, Jiangsu, China). Each group had 12 hens.

2.6.3. RNA extraction and RT-qPCR

The RNA of the tissues was extracted from eight hens randomly selected in each treatment as mentioned in 2.6.2. The RNA extraction from the duodenum and kidney was performed using RNAiso plus (Takara, Dalian, Liaoning, China) according to manufacturer's protocols, then cDNA was synthesized using the PrimeScript II 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, Jiangsu, China). The RT-qPCR analysis was conducted using a HiScript II One Step RT-qPCR SYBR Green Kit (Vazyme, Nanjing, Jiangsu, China) on the StepOne Plus Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA). All primer sequences for target genes were designed using the NCBI Primer-Blast tool (Table 3). The fold changes were calculated after normalizing to the housekeeping gene β-actin, and the $2^{-\Delta\Delta Ct}$ method was used to estimate mRNA abundance (Livak and Schmittgen, 2001). Each group had 8 samples, and all experiments were performed in triplicate.

2.6.4. Uterine transcriptomics analysis

Uterine eukaryotic transcriptome (RNA-seq) analysis was performed on six hens selected in each treatment as mentioned in 2.6.3 by using the sequencing platform (NovaSeq6000, Illumina, Inc., CA, USA). The library was constructed after the sample passed quality inspection, and the PE150 mode sequencing was performed. Bioinformatics analysis was performed using BMKCloud (www.biocloud.net). The standard for differential gene screening was fold change ≥2 and false discovery rate (FDR) < 0.01. The clean reads of each sample were sequenced with the specified reference genome, and then variable splicing prediction and gene structure optimization were analyzed.

Table 3
Sequences of oligonucleotide primers used for RT-qPCR.

Gene name	Primers sequence (5′ to 3′)	Access number
β-Actin	F: CATTGTCCACCGCAAATGCT R: AAGCCATGCCAATCTCGTCT	NM_205518
CALB1	F: TCCAGTTGTCAAACCACCCA R: ATACTGAGTGCGATGGTGGC	NM_205513
Atp2b1	F: TTCAGGTACTCATGTGATGGAAGG R: CAGCCCCAAGCAAGGTAAAG	NM_001397874
SLC8A1	F: CACTGCAGTCGTGTTTGTGG R: TCCAATAGGGCCGGAAGAGA	XM_046913144
VDR	F: TGCCTCCAGTCTGGCATCT R: CATGGCGTTGAAGTGGAAGC	NM_205098
SLC34A2	F: TGGGGAGAAAGAAGTGTCACAG R: AATTCATTCCTGTCCCCGGC	NM-204474
TRPV6	F: TGGAACGGACTAAGTCAGAAGTTG R: CGTTATGGCTGGGATGTTGTT	XM_040661661
FGFR1	F: TGACGTGCAGAGCATCAACT R: CGCTGTAGACCTTGCAGACA	NM_205510
FGFR3	F: TGTCACAGTGCTGAAGACGG R: AACGACACCTGCTGTCTCTTGA	NM_205509
FGFR4	F: ATTCCCCCGAGAAAAGCTGG R: GTCAAAGGTGTAGTCGGGCA	XM_015293864
Cyp24a1	F: GAGTTGAAACGACGGCCAAC R: TCATTGCAGCCCAAGGCATA	NM_001396287
PTH2R	F: GGTCTACGCTCTGGTGGATG R: GGCGATAAGCATGGCCTTTG	NM_001177575

F = forward primer; R = reverse primer; *CALB1* = calbindin 1; *Atp2b1* = ATPase plasma membrane Ca^{2+} transporting 1; *SLC8A1* = solute carrier family 8 member A1; *VDR* = vitamin D receptor; *SLC34A2* = Na^{+}-P-i cotransporter NaPi-IIb; *TRPV6* = transient receptor potential cation channel subfamily V member 6; *FGFR* = fibroblast growth factor receptor; *Cyp24a1* = recombinant cytochrome P450 24A1; *PTH2R* = parathyroid hormone 2 receptor.

2.7. Statistical analysis

The obtained results were subjected to statistical analysis. The experiment followed a completely randomized design with two treatments, with three replicates per treatment. All data were analyzed by SPSS 25.0 software (SPSS Inc., Chicago, IL, USA) to detect a difference with *t*-test. The threshold of $P < 0.05$ was used to indicate a statistically significant difference. Results in tables are expressed as mean ± standard deviation (SD), and results in figures are expressed as mean ± standard error of mean (SEM). Graphs were generated by GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA, USA).

Table 4
Effects of dietary compound *Bacillus* on laying performance and egg quality of hens in late laying period.

Item	Treatments[1]		*P*-value
	Con	Ba	
Laying rate in 435 d, %	77.85 ± 5.530[b]	81.46 ± 3.123[a]	0.023
Average egg weight, g	57.66 ± 3.839	56.89 ± 3.574	0.476
Sand-shell eggs ratio, %	45.24 ± 3.367[a]	21.43 ± 8.474[b]	0.018
Shell strength, N	31.16 ± 7.287[b]	36.69 ± 4.252[a]	0.029
Shell gloss	1.25 ± 0.887[b]	2.10 ± 0.943[a]	0.013
Egg shape index	1.32 ± 0.033	1.31 ± 0.033	0.192
Shell color	2.58 ± 0.826	2.70 ± 0.620	0.601
Protein height, mm	4.71 ± 1.092	5.20 ± 1.034	0.230
Haugh unit	66.65 ± 7.921	71.04 ± 7.876	0.169
Yolk color	7.80 ± 0.542	7.73 ± 0.928	0.818
Yolk ratio, %	26.91 ± 1.457	28.09 ± 1.909	0.160
Eggshell ratio, %	12.68 ± 1.764	12.26 ± 0.390	0.491
Shell thickness, × 10^{-2} mm	32.27 ± 4.761	31.93 ± 2.133	0.850

Data are presented as means ± SD. ($n = 20$).
[a,b] Means with different superscripts within each row are different at $P < 0.05$. *T*-test was applied to compare means.
[1] Con: control group with basal diet; Ba: basal diet with 5 × 10^8 CFU/kg compound *Bacillus*.

3. Results

3.1. *Compound* Bacillus *improved laying performance of hens*

The laying rate was increased with dietary supplementation of compound *Bacillus* (Table 4, $P = 0.023$). According to the anatomical results (15 hens in a group) at the end of the experiment (435 d old, 8-week treatment), we found that the ovaries and uterus of eight hens in the Con group had degenerated, accounting for 53.33%, whereas only one hen's ovary and uterus in the treatment group degenerated, accounting for 6.67%. In the follow-up feeding, the hens in the Con group were eliminated at the age of 575 d, whereas the hens in the Ba group were eliminated at the age of 645 d, extending the laying period of laying hens for 10 weeks These results indicated that the aging of reproductive organs was delayed and the laying period of hens was prolonged, thereby improving the production efficiency and the comprehensive benefits of laying hens.

3.2. *Compound* Bacillus *improved eggshell quality*

Compound *Bacillus* decreased the ratio of sand-shell eggs and increased the quality of eggshell, including shell strength and gloss ($P < 0.05$), but did not influence the other egg quality traits (Table 4). The eggshell surfaces in Con group were rough and uneven and the luster was dim in natural light, whereas the surface of eggs in the Ba group was exquisite, the coating distributed evenly, and the light reflection points could be seen obviously (Fig. 1A). The results by the egg illuminator showed that there were less small light transmitting dots on the eggshell surface (the symbolic features of sand-shell eggs) and showed uniformly orange in the Ba group compared to the Con group (Fig. 1B). Scanning electron microscope images showed that compared to the Con group, effective layer (EL) in the Ba group was thicker, mamillary cones (MC) in the eggshell cross section were clearer at 200 × magnification, and the crystal structure of the MC and EL at 4000 × magnification was orderly. In addition, the calcite column was closely connected (Fig. 1C). The thickness and crystal structure of the EL were found to be associated with its strength (Zhang et al., 2019b). Consequently, the eggshells in the Ba group exhibited greater strength resulted from their microstructure. Meanwhile, the scanning electron microscope images of eggshell surfaces in the Con group showed more cracks (Fig. 1D), which were probably caused by the irregular distribution of the cuticular layer. The Ba group exhibited comparatively smoother surfaces and finer cracks. These results indicated that compound *Bacillus* could improve the calcium secretion, making the cuticle of the eggshell more evenly distributed.

3.3. *Compound* Bacillus *extended shelf life of eggs*

Twenty eggs were placed at room temperature (about 30 °C) for 30 d. It was found that egg weight loss in the Ba group was reduced, but shell strength, protein height and Haugh unit were increased compared to the Con group ($P < 0.05$) (Table 5), indicating that compound *Bacillus* could extend shelf life of fresh eggs.

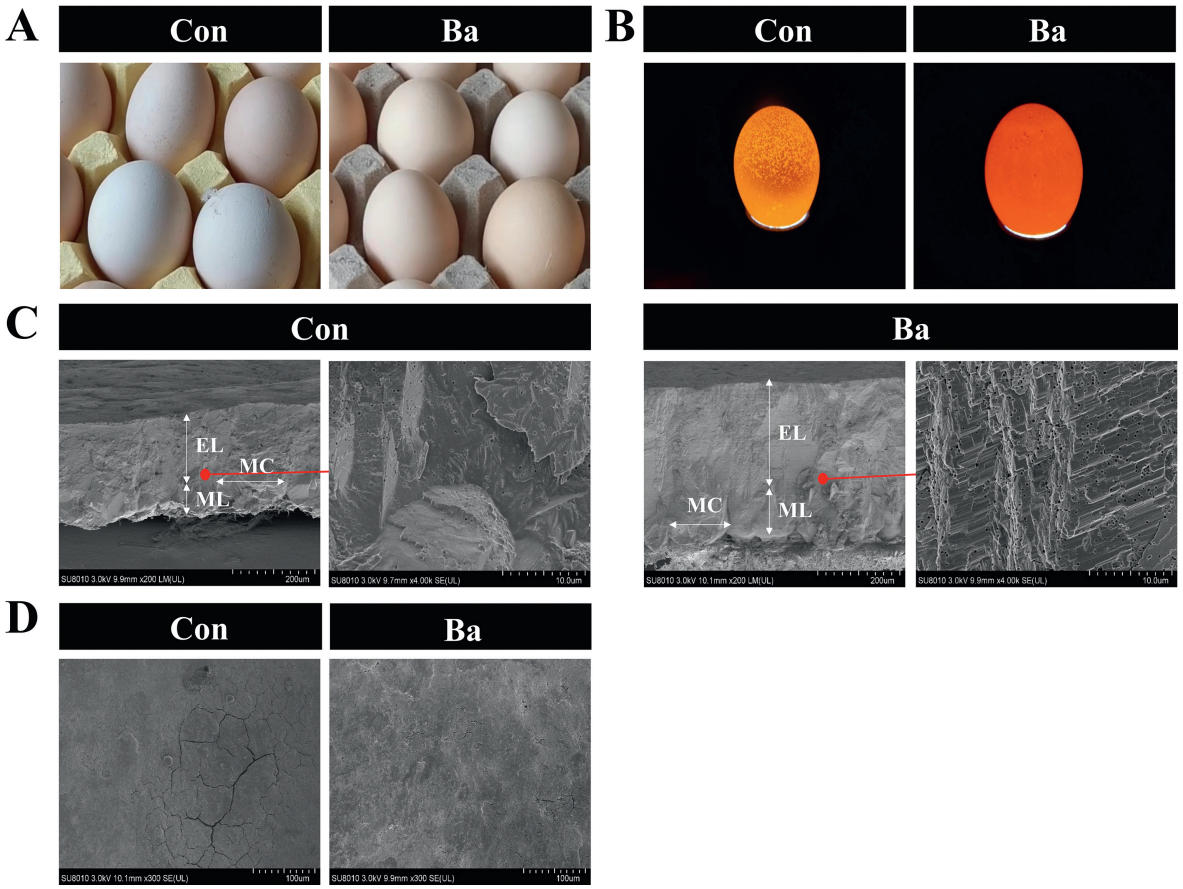

Fig. 1. Macro and micro expression of eggshell. (A) Eggshell in natural light. (B) Eggshell appearance on egg illuminator. (C) Scanning electron micrographs of eggshell cross section (Left: 200×, right: 4000×). EL = effective layer, MC = mamillary cones, ML = mamillary layer. (D) Scanning electron micrographs of eggshell surface (300×). Con: control group with basal diet; Ba: basal diet with 5×10^8 CFU/kg compound *Bacillus*.

Table 5
Effects of dietary compound *Bacillus* on egg quality after place at room temperature (about 30 °C) for 30 d.

Item	Treatments[1]		*P*-value
	Con	Ba	
Loss of egg weight, g	3.31 ± 0.605[a]	2.70 ± 0.307[b]	0.046
Shell strength, N	29.29± 8.258[b]	34.77 ± 9.732[a]	0.045
Protein heigh, mm	3.14 ± 0.544[b]	3.88 ± 1.157[a]	0.017
Haugh unit	50.84 ± 6.353[b]	60.85 ± 10.548[a]	<0.001

Data are presented as means ± SD. ($n = 20$).
[a,b] Means with different superscripts within each row are different at $P < 0.05$. *T*-tests were applied to compare means.
[1] Con: control group with basal diet, Ba: basal diet with 5×10^8 CFU/kg compound *Bacillus*.

3.4. Compound Bacillus improved metabolite composition in the yolk and albumen of eggs

The levels of TG and total cholesterol (T-CHO) in the egg yolk of the Ba group were decreased (Fig. 2A–C, $P < 0.05$). The results of metabolites in the yolk showed that the first principal component (PC1) accounted for 19.88% of the total variation (Fig. 2D), and the metabolites of the two groups were differentiated. Among a total of 6449 metabolites detected in the yolk, there were 230 different metabolites between the two groups ($P < 0.01$, fold change >1 & VIP >1), including 180 metabolites up-regulated and 50 metabolites down-regulated by compound *Bacillus* (Fig. 2E). Most of the up-regulated metabolites were organic acids and their derivatives, whereas most of the down-regulated metabolites were benzene ring compounds (Fig. 2F). Compared with the Con group, the content of p-cresol sulfate, [2-hydroxy-6-methoxy-4-(prop-2-en-1-yl)phenyl]oxidanesulfonic acid, sulfonic acid, lamellosterol acid, 3s-methyl-2-oxo-pentanoic acid, and other beneficial fatty acids in the egg yolk in the Ba group increased ($P < 0.05$), whereas the mean proportion of felbamate, glycyl-phenylalanine, glycyl-lysine, imiprothrin, and hexanoyl carnitine, was decreased ($P < 0.05$). Specifically, the metabolites with the highest increases in the Ba group were thymol sulfate ($C_{10}H_{14}O_4S$) of organic sulfonic acids and their derivatives (by 91-fold), 2-hydroxy-6-methoxy-4-(prop-2-en-1-yl) phenyl] oxidanesulfonic acid ($C_{10}H_{12}O_6S$) (by 65-fold), 1-methoxy-3-(4-hydroxyphenyl)-2e-propenal 4′-glucoside ($C_{16}H_{22}O_7$) of organooxygen compounds (by 59-fold) and [3-(4-methoxyphenyl)propoxy]sulfonic acid ($C_{10}H_{14}O_5S$) of phenol ethers (by 31-fold). Whereas, uridine monophosphate (ump) and tyrosol 4-sulfate (T4S) of organic sulfuric acids and derivatives were decreased by 15-fold and 22-fold, respectively (Fig. 2H). It was found that the different metabolites in the yolk contents were enriched in the metabolic pathways of biosynthesis of arginine and the other amino acids, as revealed by the enrichment analysis of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (Fig. 2I).

In the albumen, PC1 accounted for 22.21% of the total variation (Fig. 3A), and the metabolites of the two groups were distinct. Among a total of 6000 metabolites in the albumen, there were 135 different metabolites between the two groups ($P < 0.01$, fold change >1 & VIP >1), including 125 metabolites up-regulated and 10 metabolites down-regulated in the Ba group (Fig. 3B). Most of the up-regulated metabolites were lipids, lipid-like molecules, and organic nitrogen compounds, and most of the down-regulated metabolites were organic oxygen compounds (Fig. 3C). Compared with the Con group, the levels of beneficial metabolites such as n-stearoyl gab a, neoacrimarine b, prolylleucine, hordatine a, erythronic acid, hydroxydecanoic acid, auramine, and other metabolites of the albumen in the Ba group were increased ($P < 0.05$), whereas sulfate, 2-hydroxy capric acid, artocarpin, and suberic acid were decreased ($P < 0.05$). There was relatively little change in differential metabolites in the albumen, with the most up-regulated being glycerolipids in lipids and lipid molecules. In the albumen, $C_{21}H_{42}NO_7P$ increased by 4-fold and $C_{23}H_{44}NO_7P$, $C_{28}H_{28}N_9O_2P$, $C_{29}H_{40}NO_5PS_2$, $C_{21}H_{44}NO_7P$, $C_{24}H_{44}NO_9P$ were all upregulated by more than 3-fold. Among the downregulated metabolites, alpha-carboxyethylhydroxychroman (α-CEHC) glycine ($C_{18}H_{25}NO_5$) and pgf2alpha (PGF2α) dimethyl amide (C22H39NO4) were decreased by 3-fold and 2-fold, respectively (Fig. 3E). It was found that the different metabolites in the albumen were enriched in the metabolic pathways of biosynthesis of secondary metabolites, microbial metabolism in diverse environments, ATP-binding cassette (ABC) transporters and biosynthesis of plant secondary metabolites, as shown by the enrichment analysis of KEGG pathways (Fig. 3F).

3.5. Compound Bacillus regulated the balance of calcium and phosphorus metabolism

The key indicator for phosphate and calcium metabolism is serum 25HVD (Cheng et al., 2020). Compared to the Con group, the contents of Ca, 25HVD3, and PTH in serum of the Ba group were increased ($P < 0.05$), whereas T4 was decreased ($P = 0.048$) (Fig. 4A). As we all know, both 25HVD3 and PTH could increase calcium level in the serum, and T4 is positively associated with bone metabolism (Williams and Bassett, 2018), so it was suggested that the compound *Bacillus* could promote the absorption and reabsorption of calcium by regulating the levels of 25HVD3, T4, and PTH to mobilize bone calcium to increase blood calcium, reduce blood phosphorus, and maintain the balance between calcium and phosphorus.

According to Gloux et al. (2019), hens exhibit a greater percentage of calcium and phosphorus absorption in the proximal parts of the intestine compared to the distal parts. The genes associated with intestinal calcium uptake are ATPase plasma membrane Ca^{2+} transporting 1 (*Atp2b1*) and the receptor of parathyroid hormone 2 (*PTH2R*). The function of vitamin D is regulated by vitamin D receptor (*VDR*) and recombinant cytochrome P450 24A1 (*Cyp24a1*), which play an important role in calcium mobilization, absorption, and homeostasis (Hui et al., 2021; Tajiri et al., 2020). Phosphate absorption is facilitated by Na^+-P-i cotransporter NaPi-IIb (*SLC34A2*) and fibroblast growth factor receptor 1 (*FGFR1*) (Ren et al., 2020). In this study, the mRNA expression of *FGFR1* and *VDR* in the duodenum, associated with calcium and phosphorus metabolism, was reduced ($P < 0.05$) in the Ba group compared to the Con group. Additionally, the mRNA expression of *ATP2b1*, *SLC34A2*, and *PTH2R* was increased in the Ba group ($P < 0.05$, Fig. 4B). The above results indicated that compound *Bacillus* could promote the balance between calcium and phosphorus metabolism by regulating ATPase responsible for energy supply, phosphate transporters, and hormone receptors to improve the transport of calcium and phosphorus in the intestine. The kidney is one of the most important organs for calcium and phosphorus metabolism. The balance of calcium and phosphorus metabolism is closely regulated by the complex interaction between intestinal absorption, renal excretion, and reabsorption processes. The mRNA expression of *ATP2b1, SLC34A2, FGFR3, Cyp24a1, VDR,* and *PTH2R* in the kidney of the probiotic group were reduced compared with the Con group ($P < 0.05$, Fig. 4C), and the mRNA expression of *FGFR4* showed a downward trend as well ($P = 0.053$). *FGFR3, Cyp24a1, VDR*, and *PTH2R* are associated with vitamin D metabolism, which indicated that the metabolic pattern of vitamin D_3 was changed. The decreased expression of *ATP2b1* and *SLC34A2* implied that the active transport of calcium and phosphorus was weakened.

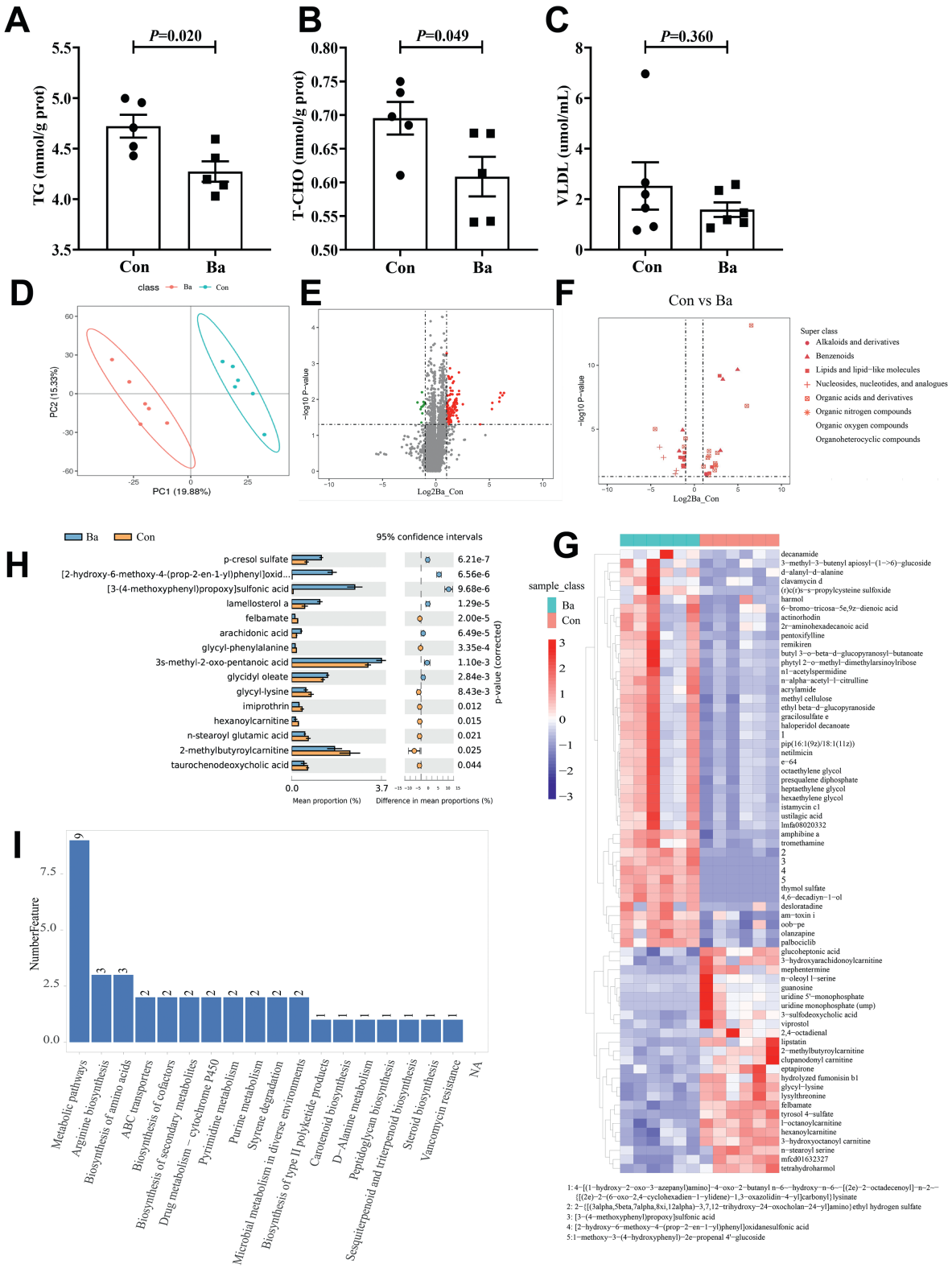

Fig. 2. Metabolite composition in the yolk. ($n = 6$). (A-C) Chemical composition of egg yolk. TG = triglycerides; TCHO = total cholesterol; VLDL = very low density lipoprotein. Data are presented as means ± SEM. (D) Principal component analysis (PCA) score plots. (E) Volcanic map. (F) Main differential metabolites in volcanic map. (G) Heat map of yolk. (H) Main differential metabolites. (I) KEGG pathway enrichment analysis. Con: control group with basal diet; Ba: basal diet with 5×10^8 CFU/kg compound *Bacillus*.

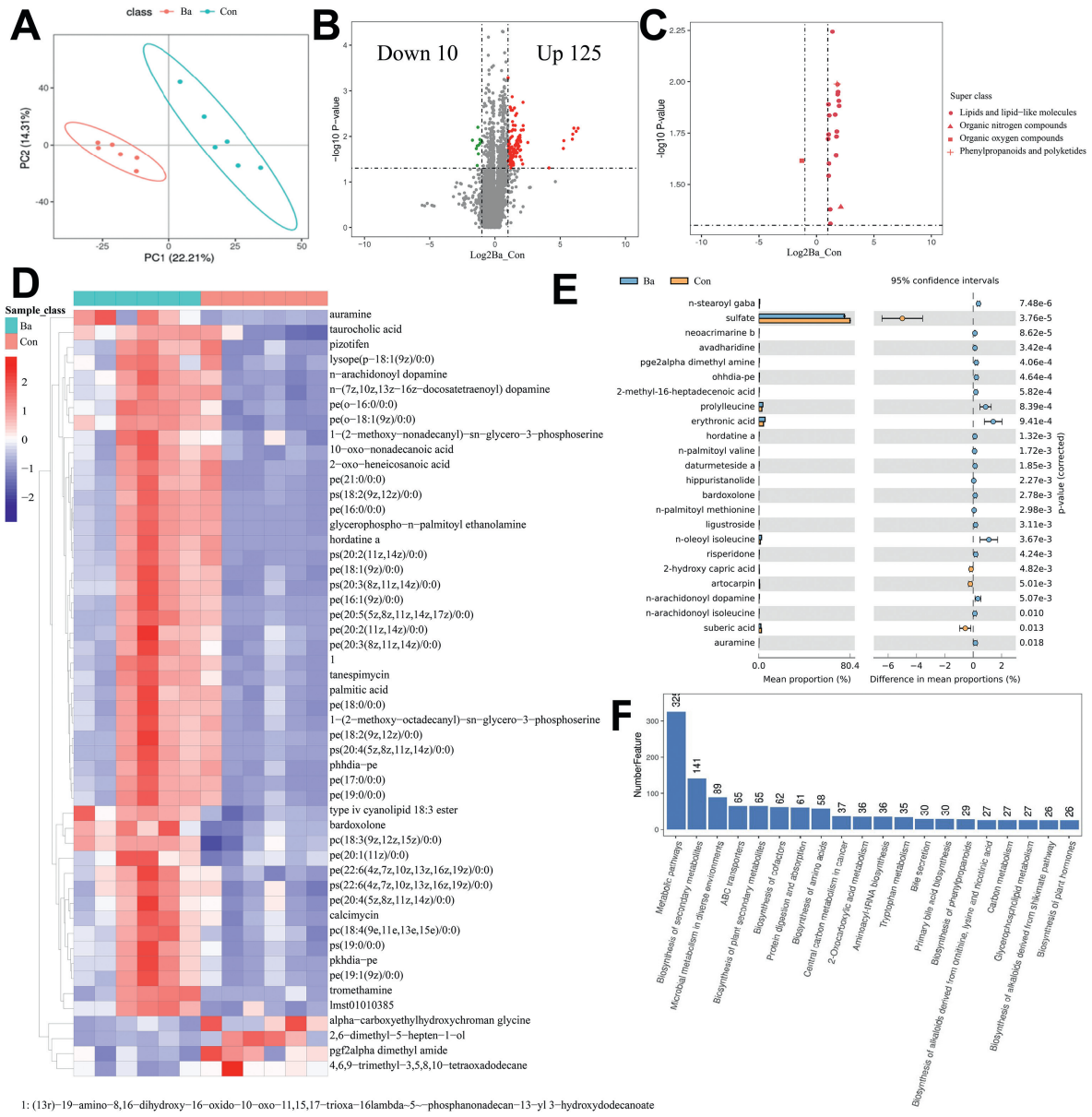

Fig. 3. Metabolite composition in the albumen. ($n = 6$). (A) Principal component analysis (PCA) score plots. (B) Volcanic map. (C) Main differential metabolites in volcanic map. (D) Heat map of yolk. (E) Main differential metabolites. (F) KEGG pathway enrichment analysis. Con: control group with basal diet; Ba: basal diet with 5×10^8 (CFU)/kg compound *Bacillus*.

3.6. *Compound* Bacillus *regulated the uterine transcriptome*

A library of 12 tissue samples from laying hens was constructed, resulting in 81.43 GB of clean data after sequencing quality control. The differences in clean reads between the 12 samples were small, and the Q30 of all samples was not less than 94.06%, and the guanine and cytosine (GC) contents of the libraries ranged from 46.48% to 50.37%, indicating that RNA sequence results were reliable and could be used for subsequent analysis (Table S1). Clean data were compared with the chicken reference genome (GRCg6a) using HISAT2. It was found that the comparison efficiency of reads for each sample and the reference genome was between 90.87% and 96.37%. A total of 3879 new genes were discovered using computational method StringTie to splice mapped reads and compare them with the original genome annotation information, based on the selected reference genome sequence. BMKCloud was used for difference analysis, and the screening criteria were FDR = 0.01, fold change = 2. The results showed that there were 38 highly significant different genes ($P < 0.01$) and the expression levels of which were decreased or increased by 2-fold. Compared with the Con group, there were 23 up-regulated genes including breast carcinoma amplified sequence 1 (*BCAS1*), LON peptidase N-terminal domain and ring finger 3 (*LONRF3*), core 1 β1,3-galactosyltransferase 1 (*C1GALT1*), joining chain of multimeric IgA and IgM (*JCHAIN*), transcription factor CP2 like 1 (*TFCP2L1*), ELL associated factor 2 (*EAF2*), interferon regulatory factor 4 (*IRF4*), 3-hydroxy-3-methylglutaryl-CoA reductase (*HMGCR*), phosphatidylinositol-4-phosphate 5-kinase type 1 beta (*PIP5K1B*), DOP1 leucine zipper like protein B (*DOP1B*), insulin induced gene 1 (*INSIG1*), avidin (*AVD*), kruppel like factor 13 (*KLF13*), ChaC glutathione specific gamma-glutamylcyclotransferase 1 (*CHAC1*), SEC11 homolog C (*SEC11C*), biliverdin reductase A (*BLVRA*), methylenetetrahydrofolate dehydrogenase 2 (*MTHFD2*), leucine rich

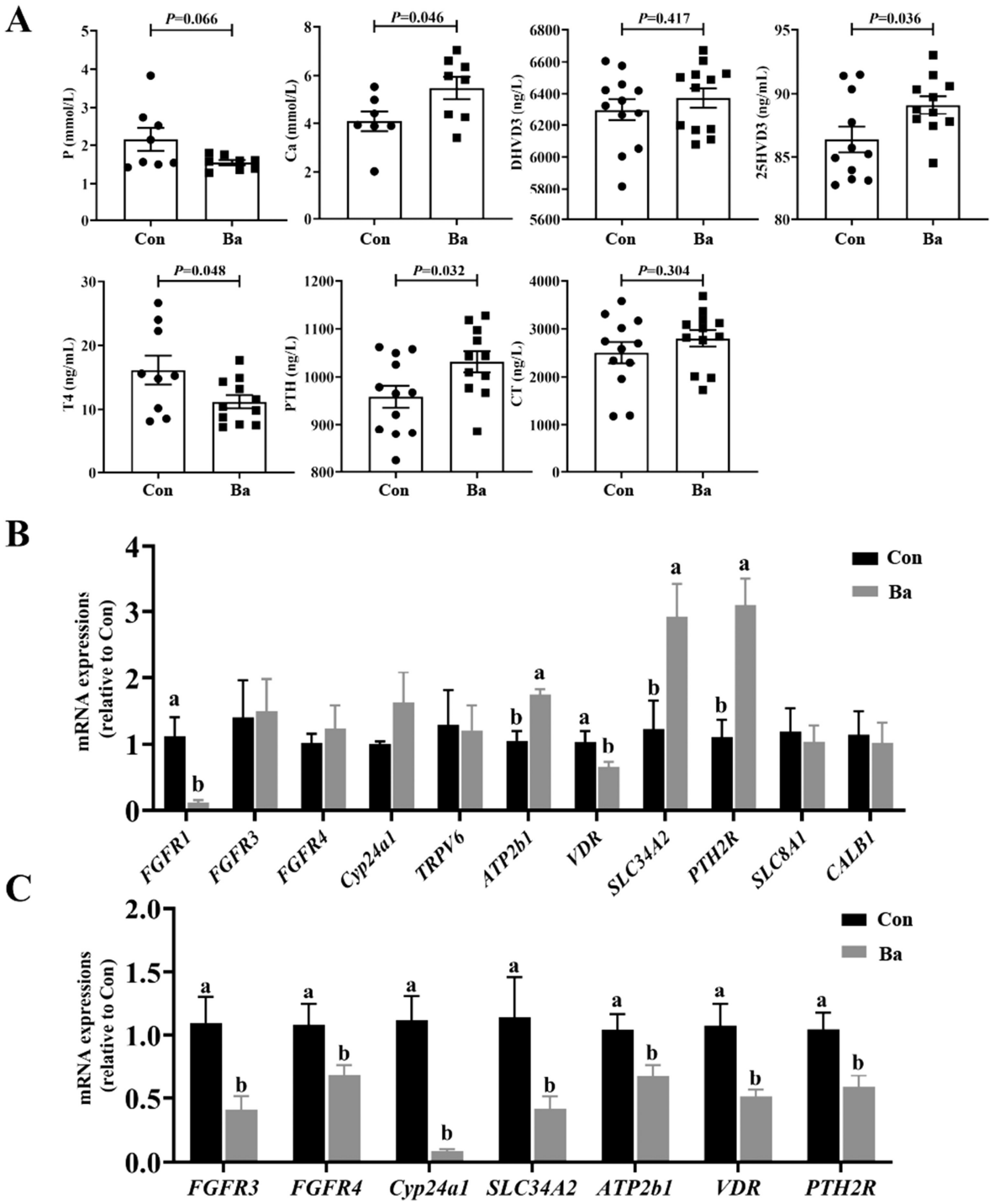

Fig. 4. Balance of calcium and phosphorus metabolism. (A) Serum calcium, phosphorus and hormones ($n = 12$). P = phosphorus; Ca = calcium; DHVD3 = 1, 25-dihydroxy vitamin D_3; 25HVD3 = 25-hydroxy vitamin D_3; T4 = thyroid hormone; PTH = parathyroid hormone; CT = calcitonin. (B) Relative mRNA expression of calcium and phosphorus metabolism in duodenal ($n = 8$). (C) Relative mRNA expression of calcium and phosphorus metabolism in kidney ($n = 8$). Con: control group with basal diet; Ba: basal diet with 5×10^8 (CFU)/kg compound *Bacillus*. Letters indicate significant differences of $P < 0.05$. Data are presented as means ± SEM.

repeat neuronal 1 (*LRRN1*), squalene epoxidase (*SQLE*), sestrin 2 (*SESN2*), DNA-damage-inducible transcript 4 (*DDIT4*), *ENSGALG00000048936*, and *ENSGALG00000023920* and 15 down-regulated genes including matrix metallopeptidase 17 (*MMP17*), semaphorin 3D (*SEMA3G*), serum/glucocorticoid regulated kinase 1 (*SGK1*), collagen type X alpha 1 chain (*COL10A1*), NIMA related kinase 3 (*NEK3*), ras-like protein family member 11A-like (*RASL11A*), integrin subunit beta 5 Gene (*ITGB5*), mitotic spindle organizing protein 2B (*MZT2B*), *RAD9*, achaete-scute homolog 3 (*ASCL3*), LOC107050147, gallus_gallus_newGene_1303, gallus_gallus_newGene_4338, gallus_gallus_newGene_5602, and gallus_gallus_newGene_9047, which were visualized in volcano plot and heatmap (Fig. 5A–B). These genes are associated with the cell proliferation: *BCAS1* (Kuo et al., 2022) , *C1GALT1* (Kuo et al., 2021), *KLF13* (Chen

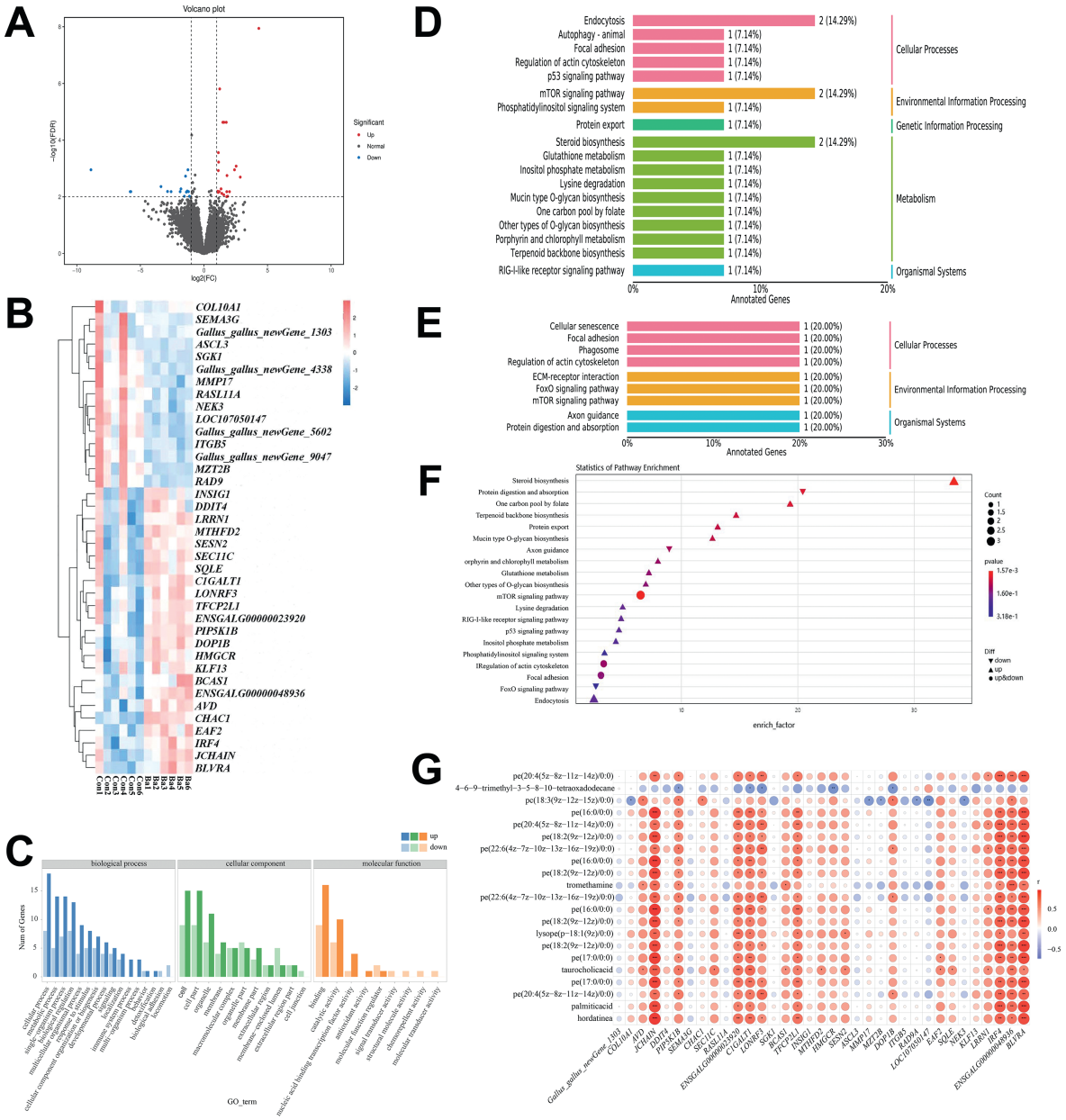

Fig. 5. Uterine transcriptomics. ($n = 6$). (A) Volcano map. (B) Heat map. (C) Differentially expressed genes of GO annotation. (D) Up-enrichment in KEGG. (E) Down-enrichment in KEGG. (F) Pathway analysis of differentially expressed genes of uterus of KEGG. (G) Correlations between uterine transcriptome and albumen metabolome (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). Con: control group with basal diet; Ba: basal diet with 5×10^8 CFU/kg compound *Bacillus*.

et al., 2022), *LRRN1* (Zhang et al., 2021a), oxidative stress *LONRF3* (Prchal et al., 2023), *PIP5K1B* (van den Bout et al., 2013), *CHAC1* (Nomura et al., 2020), *BLVRA* (Choi et al., 2023), *MTHFD2* (Shin et al., 2017), *SESN2* (Liu et al., 2021), and immunity *IRF4* (Li et al., 2021), *BLVRA* (Y. Zhang et al., 2018), *SQLE* (You et al., 2022), *DDIT4* (Ho et al., 2020), *MMP17* (Martin-Alonso et al., 2021), and *SEMA3G* (Zhou et al., 2012). It was suggested that changes in these gene expression levels can affect the systemic health, resulting in improved production efficiency and egg quality.

Gene Ontology (GO) annotation includes biological processes, molecular functions, and cellular components. Based on the results of gene GO annotation, a total of 38 differentially expressed genes in the uterus were classified into the three-level classification system of the GO database (Fig. 5C). The main functional items associated with the differentially expressed genes can be intuitively understood. Among them, the up-regulated and down-regulated genes in biological process were mainly concentrated in cellular process, metabolic process, single-organism process, and biological regulation. The up-regulated and down-regulated genes on the cellular component were mainly concentrated in the cell, cell part, organelle, and membrane. In terms of molecular function, mRNA about binding, catalytic activity, nucleic acid binding transcription factor activity, and antioxidant activity were up-regulated. On the other hand, the genes of molecular function regulation, signal transducer activity, structural molecular activity, chemorepellent activity, and molecular transducer activity were mainly found to be down-regulated.

The KEGG enrichment analysis found that the differentially expressed mRNA was associated with 24 KEGG pathways, and the specific related pathways are shown in Tables S2 and S3. The up-

regulated genes were mainly concentrated in the pathways associated with cellular processes, metabolism, environmental information processing, genetic information processing, organismal systems, and metabolism including amino acid metabolism, carbohydrate metabolism, vitamin metabolism, and lipid metabolism (Fig. 5D). Down-regulated genes were enriched in the pathways of protein digestion and absorption, axon guidance, FoxO signaling pathway, cellular senescence, phagosome, and ECM-receptor interaction (Fig. 5E). The co-enrichment pathways of up-regulated and down-regulated genes included the mTOR signaling pathway, focal adhesion, regulation of actin cytoskeleton and other biological pathways. The differentially expressed genes were enriched in the mTOR signaling pathway (enrich factor [EF] = 6.49), one carbon pool by folate (EF = 19.35), protein digestion and absorption (EF = 20.43), and steroid biosynthesis (EF = 33.43) signal pathway (Fig. 5F, $P < 0.05$). Among them, the gene expression of *SESN2* and *DDIT4* associated with the mTOR signaling pathway were up-regulated, but *SEMA3G* was down-regulated. *BCAS1* and *SQLE* genes associated with steroid biosynthesis were up-regulated. The genes related to metabolism such as *CHAC1, C1GALT1, PIP5K1B, MTHFD2, BCAS1* and *SQLE* were up-regulated, indicating that the metabolic activity of the uterus was increased.

3.7. Correlations between the uterine transcriptome and albumen metabolites

Because the uterus where the albumen forms is very close to the oviduct, there may exist a certain relationship between metabolism in the uterus and metabolites in the albumen of eggs. The Pearson correlation was done between 35 uterine differential genes which could be annotated in GO consortium and 21 albumen metabolites of eggs that were in the Human Metabolite Database (HMDB). It was found that *JCHAIN, PIP5K1B, ENSGALG00000023920, C1GALT1, LONRF3, TFCP2L1, IRF4, ENSGALG00000048936* and *BLVRA* had positive correlations with most differential metabolites in egg white ($P < 0.05$) (Fig. 5G). GO_annotation of these genes can be checked in Table S4. Interestingly, only *ENSGALG00000023920* directly acted on transport and metabolism of amino acids, while the functions of the other genes acted on improving cell vitality and proliferation (*C1GALT1*), immunity (*JCHAIN* and *IRF4*), and antioxidant capacity (*LONRF3, PIP5K1B,* and *BLVRA*). Although *TFCP2L1* and *ENSGALG00000048936* have not been reported in the literature, the former has been associated with positive regulation of growth, and the latter with microtubule cytoskeleton organization according to the GO_annotations. Thus, compound *Bacillus* have beneficial effects on the composition of albumen metabolites through enhancing gene expression related to anti-inflammatory, antioxidant, immunity, nutrient metabolism, and cell proliferation capacity in the uterus.

4. Discussion

4.1. Calcium and phosphorus metabolism and eggshell

The quality of eggshells, including shell thickness, shell strength, amount of cuticle present, etc., can affect the ability of bacteria to penetrate the eggshell, which affects the shelf life of eggs. Fragile eggshells make eggs more susceptible to microbial contamination, which shortens their storage time and causes economic losses during transportation (Sharaf et al., 2019). Previous studies have shown that probiotics can increase the average egg weight and eggshell strength and reduce the percentage of abnormal eggs (Nishiyama et al., 2021; Xiang et al., 2019). Our experimental results also confirmed that compound *Bacillus* could improve the gloss and strength of eggshells and reduce the ratio of sandpaper-shelled eggs.

The gloss of eggshells is an important appearance characteristic that can influence consumer purchasing behaviors. Because it is difficult to objectively quantify, there are few studies about gloss of eggs. The smooth cuticle of the eggshell can produce a mirror-like luster, reduce the obstruction of gas exchange, and its high reflection of light will also help reduce the damage from solar radiation to the embryo (Igic et al., 2015). The glossy appearance of eggshells is mainly determined by the cuticle, which is composed of calcite, calcium phosphate, and organic compounds such as proteins, lipids, polysaccharides, and pigments. The higher the coverage rate of the cuticle and the lower the surface roughness, the better the luster (Li et al., 2019). Our study developed a scoring criterion for eggshell gloss (Table 2) and was the first to confirm that probiotics could enhance eggshell gloss according to this criterion.

It is generally believed that the strength of eggshell is closely associated with metabolic balance between calcium and phosphorus, especially intestinal absorption and renal excretion. The absorption of calcium and phosphorus by hens is mainly located in the proximal intestine because of the high expression of calcium and phosphorus transporters (Wang et al., 2022), including *Atp2b1, VDR, SLC34A2*, and *FGFR1.* ATPase plasma membrane Ca^{2+} transporting 1 (*Atp2b1*), which is a gene associated with intestinal calcium uptake (Gloux et al., 2019), and the mRNA level of vitamin D receptor (*VDR*) could increase the weakened calcium absorption capacity during the later laying period (Hui et al., 2021). Fibroblast growth factor receptor 1 (*FGFR1*) and Na^{+}-P-i cotransporter NaPi-IIb (*SLC34A2*) participate in phosphate absorption, and the former is an indicator of dietary phosphorus restriction and causes acute adaptive up-regulation of the latter to increase absorption of phosphate (Ren et al., 2020). Probiotics could improve apparent digestibility of calcium and phosphorus, thereby increasing their contents in serum and maintaining calcium and phosphorus balance (Zhang et al., 2020). In this experiment, the resultant decrease in the expression of *FGFR1* and *VDR* and increase in the expression of *Atp2b1*, *SLC34A2,* and *PTH2R* proved that the compound *Bacillus* could promote the absorption of calcium and phosphorus in the duodenum. After absorption, calcium and phosphorus are mainly transported through the bloodstream to various parts of the body, which affect the balance of calcium and phosphorus metabolism in the whole body via related hormones (PTH, CT, and activated vitamin D_3) (Cheng et al., 2020). Both PTH and activated vitamin D_3 could promote the absorption of calcium and phosphorus in the intestine and reabsorption in the kidney and then effectively improve the quality of eggshell through improving calcium levels in the blood (Adhikary et al., 2015; Phelps et al., 2014). Zhang et al. (2019a) found that 1,25-dihydroxy vitamin D_3 could affect the ultrastructure of the papillary layer by regulating gene expression in the uterus. Furthermore, CT could promote urinary phosphorus excretion and reduce reabsorption of calcium in the kidney. In this study, it was found that compound *Bacillus* regulated calcium and phosphorus metabolism in laying hens by increasing the concentration of active vitamin D_3 and PTH and decreasing the content of T4 to increase serum calcium content and provide a greater reserve of calcium to produce high-quality eggshells. This conjecture also supported Kwiatkowska's conclusion (2017) that probiotics could stimulate calcium accumulation in poultry. In addition, the genes mentioned in the duodenum such as *ATP2b1, SLC34A2,* and *VDR* also exist in the kidneys. The metabolism of vitamin D_3 by *FGFR3, Cyp24a1, PTH2R* can affect excretion and reabsorption of calcium and phosphorus in the kidney and is transcriptionally regulated by interactions among *FGF23*, DHVD3 and PTH. *PTH2R* is the receptor of parathyroid hormone 2 that can convert vitamin D into an active form, and *Cyp24a1* is a catabolic enzyme of vitamin D (Tajiri et al.,

2020). The concentration of *FGFR3*, which was decreased in the Ba group in renal tissue, inhibits *FGF23* signalling, thereby facilitating the production of DHVD3 in the proximal tubule, which ultimately results in an increase in serum calcium levels (Liu et al., 2008; Mutsaers et al., 2014). The decrease of expression of these genes suggested that compound *Bacillus* may have enhanced passive transport and reduced the need for active transport, or passive transport had already satisfied the calcium and phosphorus requirements in the blood because most of the reabsorption of calcium in proximal tubule is passive (Curry and Yu, 2019), which needs to be further investigated. To sum up, compound *Bacillus* may mainly regulate the dynamic balance of calcium and phosphorus in the blood by increasing the concentration of active vitamin D_3, and reducing carrier mediated transport in the kidney to prevent hypercalcemia.

The uterus is the organ where eggs are formed and undergo calcification initiation, linear deposition, and late calcification to complete the eggshell calcification process (Zhang et al., 2021b). Limited research has been conducted on the effects of probiotics on the production of sandpaper-shelled eggs which may be produced during the process of eggshell formation due to uneven calcium deposition caused by uterine mucosa shedding, epithelial cell damage, and the degradation of the organs of aging laying hens or metabolic imbalance of calcium and phosphorus (Feng et al., 2020; Zhang et al., 2022b). However, although the experiment confirmed that there were effects on calcium and phosphorus metabolism in the blood, duodenum, and kidney, the transcriptome sequencing of the uterus did not reveal any changes in genes directly associated with calcium and phosphorus metabolism. The anatomical results have shown a decrease in the rate of uterine and ovarian degeneration, so we postulated that in addition to the metabolic balance of calcium and phosphorus, the improvement in eggshell quality may also be associated with slower aging of laying hens and a younger and more vibrant uterus. Transcriptome analysis showed that the expression of the gene integrin subunit beta 5 (*ITGB5*) related to cell aging in the uterus was reduced, which is associated with the KEGG pathways of protein digestion and absorption, axon guidance, and phagosome. Although functional research on *ITGB5* in poultry is lacking, in mammals Chung et al. (2016) it was found that the high expression of *ITGB5* could enhance the adhesion between endometrial cells. Additionally, Zhang et al. (2018) found that *ITGB5* expression may increase during female aging. Furthermore, the expression of *SESN2* genes, which are associated with both the p53 signaling pathway and mTOR signaling pathway was increased. Previous studies in humans and mice have shown that p53 induced *SESN2* expression, which could target mTORC1 signaling through an AMP-activated protein kinase (AMPK) independent mechanism to promote mTORC1 translocation to lysosomes to downregulate mTORC1 signaling and increase pAMPK activity to alleviate inflammation (Deng et al., 2016). Historical research found that enhancing tight junctions in the uterine mucosa could effectively improve mucosal barrier function, and inflammatory reactions would disrupt the gene and protein expression of factors associated with eggshell formation and lead to eggshell deformities (Nii et al., 2023, 2018; 2014). *Bacillus* has been found to inhibit the inflammatory response in the uterus (Jung et al., 2023). Therefore, it is believed that compound *Bacillus* could enhance the anti-inflammatory ability and mucosal barrier function in the uterus, thereby improving the quality of eggshells. Furthermore, the differentially expressed genes related to metabolism in KEGG_pathway_annotation such as carbohydrate metabolism, amino acid metabolism, glycan biosynthesis and metabolism, metabolism of cofactors and vitamins, lipid metabolism, metabolism of terpenoids and polyketides were all upregulated. The above results were consistent with the upregulated metabolites in the albumen. Interestingly, we found some upregulated genes, such as *BCAS1*, *C1GALT1*, *KLF13*, and *LRRN1* could enhance cell proliferation and migration ability, and high expression of these genes was generally associated with cancer progression in historical studies. Therefore, it was speculated that the enhancement of cell proliferation ability may promote the vitality of organ tissues and could delay aging. In summary, gene expression levels suggested that compound *Bacillus* increased uterine metabolic activity, slowed uterine aging and reduced the inflammatory response in aging laying hens.

4.2. Differences in metabolites of the yolk and albumen of eggs

Our experiment confirmed that probiotics could reduce the levels of cholesterol and triglycerides in eggs. Historical studies have shown that probiotics could affect the body's cholesterol and lipid metabolism, reducing the T-CHO concentration in blood (Guo et al., 2011). Application of probiotics could extend to egg products, reducing the T-CHO in eggs. We speculated that a metabolite regulated by probiotics could affect gene expression in the body. For example, inhibiting lipid metabolism could lead to a decrease in T-CHO in egg yolks (Wei et al., 2022). The results of transcriptome analysis showed that compound *Bacillus* could regulate the expression of genes associated with cholesterol metabolism in the uterus such as *HMGCR, SQLE, KLF13, INSIG1*, and *SESN2*. Among these genes, *HMGCR* (Menzies et al., 2018) and *SQLE* (Zou et al., 2022) are rate limiting enzymes for cholesterol biosynthesis, and *KLF13* can promote cholesterol biosynthesis and adipocyte differentiation (Chen et al., 2022). *INSIG1* can inhibit cholesterol biosynthesis by degrading *HMGCR*, which reduces the transcription of adipogenic genes (Chen et al., 2021), while *SESN2* can reduce lipid biosynthesis (Wang et al., 2021). Therefore, changes in expression of the above genes can modulate metabolites in egg products.

Metabolomics analysis revealed that compound *Bacillus* increased beneficial fatty acid content and metabolites with differential level in the egg yolk, which were enriched in arginine biosynthesis and amino acid biosynthesis. Notably, thymol sulfate and oxidanesulfonic acid exhibited the largest changes, with increases of 91- and 65-fold, respectively, both of which possess antibacterial effects. The former is a metabolite of thymol which has an inhibitory effect on pathogens such as *Salmonella*, and the latter with different ligands could be used to treat bacterial infections, which may act to extend the shelf life of egg products. Yan et al. (2020) also confirmed that high levels of antimicrobial proteins in eggs can resist pathogen invasion. The largest changes in the decreasing metabolites were T4S (22-fold) and UMP (15-fold). The decrease in T4S may be due to increased antioxidant capacity (details in section 3.6) causing a decrease in reactive oxygen species, resulting in a decrease in the metabolism of tyrosol which is considered a potential antioxidant (Lee et al., 2016). Meanwhile, the decrease in UMP, which affects fatty acid metabolism, would reduce total cholesterol and triglycerides in egg yolk (Zhang et al., 2019a) which was confirmed by Pearson correlation analysis (UMP & TCHO, $R^2 = 0.751$, UMP & TG, $R^2 = 0.822$; $P = 0.008$) in this study.

In addition, the number of differential metabolites of the albumen is minor; the main upregulation products of which were glycerophospholipids in lipid and lipid-like molecules, which are non-volatile compounds that undergo thermal degradation to produce a rich aroma and are important flavor compounds in food (Sun et al., 2022). Glycerophospholipids is the most abundant lipid group in eggs, and these metabolites are important nutrients that are beneficial for human health (Liu et al., 2023). The main downregulated metabolites were alpha-carboxyethylhydroxychroman (α-CEGC) glycine (3 times) and prostaglandin F2α (PGF2α)

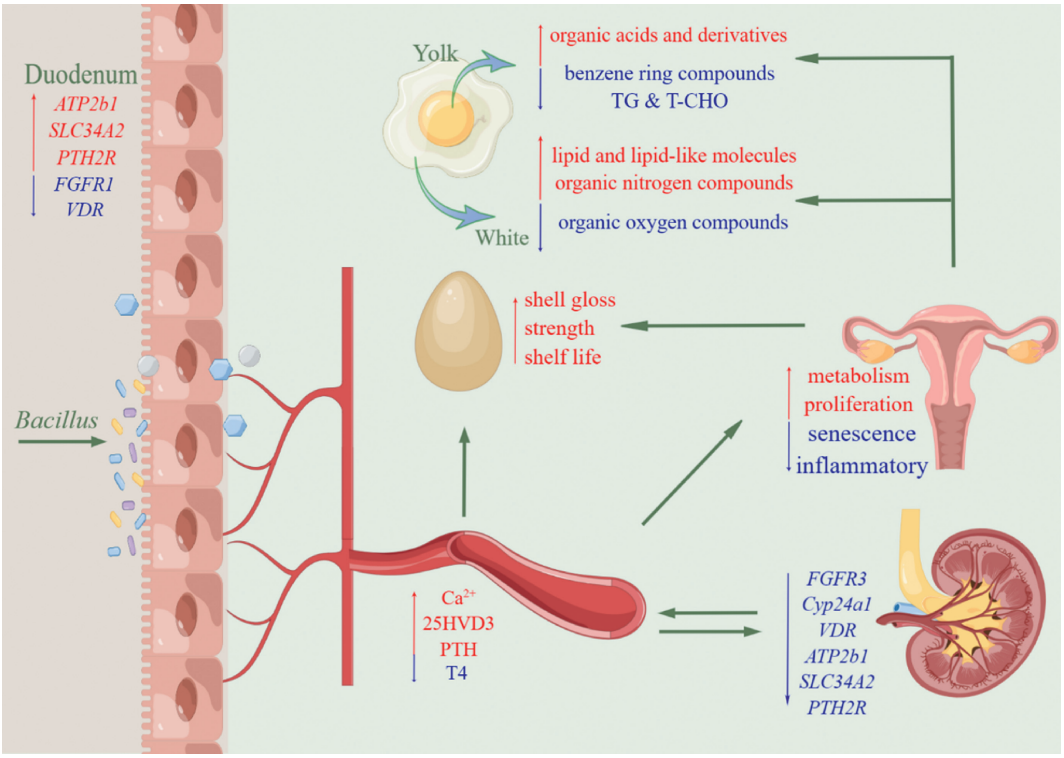

Fig. 6. Overall changes in Jingfen no. 6 laying hens. TG = triglycerides; TCHO = total cholesterol; Ca = calcium; 25HVD3 = 25-hydroxy vitamin D_3; PTH = parathyroid hormone; T4 = thyroid hormone; *ATP2b1* = ATPase plasma membrane Ca^{2+} transporting 1; *VDR* = vitamin D receptor; *SLC34A2* = Na^{+}-P-i cotransporter NaPi-Iib; *FGFR* = fibroblast growth factor receptor; *Cyp24a1* = recombinant cytochrome P450 24A1; *PTH2R* = parathyroid hormone 2 receptor.

dimethyl amide (2 times) which are both associated with the function of the uterus. α-CEGC glycine is a metabolite of α-tocopherol that has antioxidant and anti-aging properties (Johnson et al., 2012), and the reason for its downregulation may be the weakened oxidative stress and postponed aging in the laying hens, which lead to a decrease in α-tocopherol metabolism. Dimethyl amide is an antagonist of the PGF2α receptor in the endometrium, which could inhibit the contraction of uterine smooth muscle to inhibit ovulation. A low concentration of PGF2α dimethyl amide in the albumen may have been due to a high concentration of PGF2α in the uterus, therefore increasing egg production. Further, during storage, the degradation of nutrients such as amino acids, fatty acids, nucleotides, sugars, and vitamins in eggs can result in the production of biogenic amines and other substances (Liu et al., 2022). Therefore, an increase in antioxidant and antibacterial metabolites in eggs could slow down the degradation process of the nutrients in eggs, resulting in modulations in metabolites.

5. Conclusion

In summary, dietary supplementation with compound *Bacillus* could improve the quality of eggs and prolong the laying period of hens by promoting the metabolic balance between calcium and phosphorus and cell proliferation and nutrient metabolism in the uterus (Fig. 6).

Credit author statement

Qian Jin: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Visualization. **Fei Wang:** Formal analysis, Visualization, Writing - Review & Editing. **Weisheng Ye:** Investigation, Resources. **Qi Wang:** Methodology, Validation. **Shujie Xu:** Investigation, Validation. **Shaoxiong Jiang:** Supervision, Resources. **Xiang Li:** Investigation, Validation. **Min Yue**, **Dongyou Yu**, and **Mingliang Jin**: Writing - Review & Editing. **Aikun Fu:** Supervision, Writing - Review & Editing. **Weifen Li:** Conceptualization, Supervision, Project administration, Funding acquisition.

Availability of data and material

The datasets produced and/or analyzed during the current study are available from the corresponding author on reasonable request.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2024.04.023.

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