

Effects of *Bacillus subtilis* CGMCC 1.921 on Production Performance, Hematological Parameters, Serum Biochemical Parameters, and Immunoglobulin Content in Laying Hens (Post-print)

Authors: Guo Junrui, Dong Xiaofang, Tong Jianming

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Abstract

This experiment aimed to investigate the effects of *Bacillus subtilis* CGMCC 1.921 on production performance, hematological parameters, serum biochemical parameters, and immunoglobulin content in laying hens. A total of 360 healthy 27-week-old Hy-Line Brown laying hens were selected and randomly divided into 5 groups with 6 replicates per group and 12 hens per replicate. The control group (Group 1) was fed a basal diet, while the treatment groups (Groups 2-5) were fed the basal diet supplemented with 1.0×10^5 , 1.0×10^6 , 1.0×10^7 , and 1.0×10^8 CFU/g of *Bacillus subtilis* CGMCC 1.921, respectively. The experimental period lasted for 24 weeks. The results showed that there were no significant differences among groups in laying rate, feed intake, egg weight, egg production per hen, or mortality and culling rate ($P > 0.05$), but the feed conversion ratio in Groups 2, 4, and 5 was significantly lower than that in the control group ($P < 0.05$); dietary supplementation with *Bacillus subtilis* CGMCC 1.921 significantly increased serum glucose content (in weeks 2, 3, 12, 16, and 20) ($P < 0.05$) and significantly decreased serum urea content (in weeks 2, 3, 8, and 12) ($P < 0.05$); dietary supplementation with *Bacillus subtilis* CGMCC 1.921 significantly increased blood leukocyte count (in weeks 1, 3, 4, 8, 12, 16, 20, and 24) and lymphocyte count (in weeks 2, 3, 4, 8, 12, 16, 20, and 24) ($P < 0.05$), and significantly elevated serum immunoglobulin G (in weeks 1 and 4) and immunoglobulin M content (in weeks 2, 4, 8, and 16) ($P < 0.05$). In conclusion, dietary supplementation with *Bacillus subtilis* CGMCC 1.921 reduced the feed conversion ratio, exerted certain ameliorative effects on serum glucose and urea contents and hematological parameters, and enhanced immunity in laying hens to a certain extent.

Full Text

Effects of *Bacillus subtilis* CGMCC 1.921 on Performance, Routine Blood Parameters, Serum Biochemical Parameters and Immunoglobulin Contents of Laying Hens

GUO Junrui, DONG Xiaofang*, TONG Jianming

(Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing 100193, China)

Abstract: This study was conducted to investigate the effects of *Bacillus subtilis* CGMCC 1.921 on performance, routine blood parameters, serum biochemical parameters and immunoglobulin contents of laying hens. A total of 360 healthy 27-week-old Hy-Line brown laying hens were randomly allocated into 5 groups with 6 replicates per group and 12 birds per replicate. The control group (Group 1) was fed a basal diet, while the experimental groups (Groups 2–5) were fed the basal diet supplemented with 1.0×10^8 , 1.0×10^7 , 1.0×10^6 and 1.0×10^5 CFU/g of *Bacillus subtilis* CGMCC 1.921, respectively. The experiment lasted for 24 weeks. The results showed that there were no significant differences in egg production rate, feed intake, egg weight, egg weight per hen, or mortality among all groups ($P > 0.05$). However, Groups 2, 4, and 5 had significantly lower feed-to-egg ratios compared with the control group ($P < 0.05$). Dietary supplementation with *Bacillus subtilis* CGMCC 1.921 significantly increased serum glucose content (at weeks 2, 3, 12, 16, and 20) ($P < 0.05$) and significantly decreased serum urea content (at weeks 2, 3, 8, and 12) ($P < 0.05$). Supplementation also significantly increased blood leukocyte counts (at weeks 1, 3, 4, 8, 12, 16, 20, and 24) and lymphocyte counts (at weeks 2, 3, 4, 8, 12, 16, 20, and 24) ($P < 0.05$), and significantly elevated serum immunoglobulin G content (at weeks 1 and 4) and immunoglobulin M content (at weeks 2, 4, 8, and 16) ($P < 0.05$). In conclusion, dietary supplementation with *Bacillus subtilis* CGMCC 1.921 reduced the feed-to-egg ratio, improved serum glucose and urea contents and routine blood parameters, and enhanced the immune function of laying hens to a certain extent.

Keywords: laying hens; *Bacillus subtilis*; performance; routine blood parameters; serum biochemical parameters; immunoglobulin

Corresponding author: Associate Professor, E-mail: xiaofangd1124@sina.com

In recent years, microbial feed additives have been increasingly applied in animal production. Lactic acid bacteria are currently considered one of the earliest applied and most recognized probiotics by producers and farmers, while *Bacillus* species have gradually gained favor due to their high stability under adverse conditions such as high temperature and acidic environments. *Bacillus subtilis* was listed in the “Catalogue of Feed Additives Permitted for Use” by the Ministry of Agriculture in 1999. Studies have shown that dietary supplementa-

tion with *Bacillus subtilis* can enhance the activity of digestive enzymes such as protease and amylase in the intestine, improve intestinal structure, increase nutrient utilization, and promote animal growth and development. It can also inhibit pathogen colonization, enhance disease resistance, reduce the emission of harmful substances, and improve the farming environment. However, there have been no long-term continuous observations on the effects of *Bacillus subtilis* on laying hens.

This experiment aimed to investigate the effects of dietary supplementation with different levels of *Bacillus subtilis* CGMCC 1.921 on the performance, routine blood parameters, serum biochemical parameters, and immunoglobulin contents of laying hens, providing a theoretical reference for its application in laying hen diets.

1.1 Experimental Strain

The *Bacillus subtilis* CGMCC 1.921 strain used in this experiment was purchased from the China General Microbiological Culture Collection Center and produced by Cangzhou Huayu Pharmaceutical Co., Ltd., with a content of 1.105×10^1 CFU/g.

1.2 Experimental Animals and Design

A total of 360 healthy 27-week-old Hy-Line brown laying hens were randomly divided into 5 groups with 6 replicates per group and 12 birds per replicate. Group 1 served as the control group and was fed a basal diet without *Bacillus subtilis* CGMCC 1.921, while Groups 2-5 were fed the basal diet supplemented with 1.0×10^1 , 1.0×10^2 , 1.0×10^3 , and 1.0×10^4 CFU/g of *Bacillus subtilis* CGMCC 1.921, respectively. The experimental period lasted for 24 weeks. The basal diet was formulated according to the nutrient requirements for laying hens from NRC (1994). Its composition and nutrient levels are shown in Table 1.

Table 1 Composition and nutrient levels of the basal diet (air-dry basis), %

Items	Content
Ingredients	
Corn	
Soybean meal	
Soybean oil	
NaCl	
Limestone	
CaHPO	
Premix ¹⁾	
Total	
Nutrient levels²⁾	
ME/(MJ/kg)	

Items	Content
CP	
Ca	
TP	
AP	
Lys	
Met	
Met+Cys	

¹⁾ The premix provided the following per kg of diet: VA 8,000 IU, VD 3,000 IU, VE 15 IU, VK 2.0 mg, VB 2.0 mg, VB 4.0 mg, VB 4.0 mg, calcium pantothenate 12 mg, nicotinic acid 40 mg, biotin 0.1 mg, folic acid 1.0 mg, VB 0.01 mg, Fe (as ferrous sulfate) 30 mg, Cu (as copper sulfate) 6.25 mg, Zn (as zinc sulfate) 70 mg, Mn (as manganese sulfate) 63.6 mg, I (as potassium iodide) 0.4 mg, Se (as sodium selenite) 0.2 mg.

²⁾ Calculated values.

1.3 Management

A two-tier cage system was used, with each replicate consisting of two adjacent cages (80 cm × 60 cm × 50 cm), housing 6 birds per cage. Birds were fed three times daily with ad libitum access to feed and water via nipple drinkers. The lighting schedule was 16 h (06:00-22:00) with automatic temperature control, heating, and ventilation.

1.4 Measurements

1.4.1 Performance During the experimental period, egg number, egg weight, and mortality were recorded daily for each replicate. Feed consumption was recorded weekly, and remaining feed was weighed to calculate egg production rate, feed intake, egg weight, feed-to-egg ratio, egg weight per hen, and mortality rate.

1.4.2 Serum Biochemical Parameters At the start of the experiment (Week 0) and at Weeks 1, 2, 3, 4, 8, 12, 16, 20, and 24, four birds were randomly selected from each replicate after 12 h of fasting (with free access to water). Blood was collected from the wing vein into regular vacuum tubes, allowed to clot, then centrifuged at 2,000 r/min for 10 min. The supernatant was collected in EP tubes for analysis. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein (TP), albumin (ALB), total bilirubin (TBIL), glucose (GLU), urea (UREA), uric acid (UA), creatinine (CREA), triglycerides (TG), and cholesterol (CHOL) were measured using a Toshiba TBA-120FR automatic biochemical analyzer.

1.4.3 Hematological Parameters At the start of the experiment (Week 0) and at Weeks 1, 2, 3, 4, 8, 12, 16, 20, and 24, four birds were randomly selected from each replicate after 12 h of fasting (with free access to water). Blood was collected from the wing vein into 5 mL EDTA-anticoagulant vacuum tubes and 2 mL sodium citrate-anticoagulant vacuum tubes, and gently mixed. Leukocyte (WBC), lymphocyte (LYM), mid-cell (MID), neutrophil (NEU), red blood cell (RBC), and platelet (PLT) counts, as well as hematocrit (HCT) and hemoglobin (HGB) content were determined by impedance method using a Jiangxi Tekang TEK-II mini automatic three-part hematology analyzer. Erythrocyte sedimentation rate (ESR) was measured by the Westergren method using a Beijing PreeKine XC-40B automatic ESR analyzer.

1.4.4 Serum Immunoglobulin Content Blood sampling was performed as described in section 1.4.2. Serum immunoglobulin A (IgA), immunoglobulin G (IgG), and immunoglobulin M (IgM) contents were determined by double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) using chicken immunoglobulin (IgA, IgG, IgM) ELISA kits provided by Shanghai Langdun Biotechnology Co., Ltd.

1.5 Statistical Analysis

Experimental data were analyzed using SPSS 19.0. One-way ANOVA and least significant difference (LSD) multiple comparison tests were performed. The significance level was set at $P < 0.05$. Data are expressed as means \pm standard deviation.

2.1 Effects of *Bacillus subtilis* CGMCC 1.921 on Performance of Laying Hens

As shown in Table 2, dietary supplementation with *Bacillus subtilis* CGMCC 1.921 had no significant effects on egg production rate, egg weight, feed intake, egg weight per hen, or mortality during Weeks 1-24 ($P > 0.05$). However, Groups 2, 4, and 5 had significantly lower feed-to-egg ratios compared with the control group ($P < 0.05$), while Group 3 showed no significant difference from other groups ($P > 0.05$).

Table 2 Effects of *Bacillus subtilis* CGMCC 1.921 on performance of laying hens

Items	Group 1	Group 2	Group 3	Group 4	Group 5	P-value
Egg production/%	89.5 \pm 5.4	92.4 \pm 2.5	92.5 \pm 1.7	92.5 \pm 4.0	93.1 \pm 3.6	
Feed intake/(g/d)	111.4 \pm 2.0	111.5 \pm 1.5	111.0 \pm 1.7	110.7 \pm 1.6	111.2 \pm 2.6	

Items	Group 1	Group 2	Group 3	Group 4	Group 5	P-value
Egg weight/g	61.2±0.5	61.5±0.8	60.9±1.0	61.4±1.1	61.6±0.3	
Feed to egg ratio	2.04±0.10	1.96±0.04	1.97±0.03	1.95±0.04	1.94±0.04	
Egg weight per hen/kg	9.09±0.57	9.44±0.30	9.35±0.30	9.42±0.32	9.52±0.38	
Mortality/%	1.39±3.40	1.39±3.40	2.78±4.30	0.00±0.00	0.00±0.00	

In the same row, values with different small letter superscripts mean significant difference ($P < 0.05$), while with the same or no letter superscripts mean no significant difference ($P > 0.05$). The same as below.

2.2 Effects of *Bacillus subtilis* CGMCC 1.921 on Serum Biochemical Parameters of Laying Hens

As shown in Table 3, serum ALT activity in Groups 2 and 5 (Week 1) was significantly higher than in the control group ($P < 0.05$), while ALT activity in all experimental groups (Week 24) showed a decreasing trend ($P = 0.072$). For serum AST activity, only Group 3 (Week 2), Group 4 (Week 8), and Group 5 (Weeks 3 and 20) were significantly higher than the control group ($P < 0.05$).

Table 3 Effects of *Bacillus subtilis* CGMCC 1.921 on serum ALT and AST activities of laying hens, U/L

Items	Group 1	Group 2	Group 3	Group 4	Group 5	P-value
ALT						
Week 1	2.9±1.1	4.1±1.5	3.4±1.3	3.3±1.3	4.2±1.7	
Week 24	1.8±1.2	1.6±1.1	1.7±1.0	1.1±0.9	1.1±0.8	
AST						
Week 2	146±14	147±11	159±18	153±13	153±18	
Week 3	147±13	145±12	149±14	144±13	155±15	
Week 8	143±14	148±11	150±16	158±18	149±14	
Week 20	141±27	138±26	140±27	144±27	166±33	

As shown in Table 4, serum TP and GLB contents in Group 5 (Week 3) were significantly higher than in other groups ($P < 0.05$). Serum ALB content in Group 5 (Week 2) was significantly higher than in the control group and Group 3 ($P < 0.05$), while serum GLB content in Group 2 (Week 2) was significantly higher than in the control group and Groups 3 and 5 ($P < 0.05$). The serum

albumin/globulin ratio (A/G) in Group 2 (Week 2) was significantly lower than in the control group and Group 5 ($P<0.05$), and the A/G ratio in Group 5 (Week 3) was significantly lower than in other groups ($P<0.05$).

Table 4 Effects of *Bacillus subtilis* CGMCC 1.921 on serum TP, ALB, GLB content and A/G of laying hens

Items	Group 1	Group 2	Group 3	Group 4	Group 5	P-value
TP/(g/L), Week 3	41.2±4.0	41.5±7.5	40.0±5.8	41.0±7.6	51.1±5.5	<0.001
ALB/(g/L), Week 2	21.0±1.6	21.9±1.9	21.1±1.4	22.0±1.8	22.4±2.0	
GLB/(g/L), Week 2	24.2±4.3	27.6±4.2	24.9±4.4	26.0±3.6	24.6±4.3	
GLB/(g/L), Week 3	19.3±3.0	20.1±6.1	18.7±4.8	20.3±6.5	29.2±4.4	<0.001
A/G, Week 2	0.89±0.12	0.80±0.08	0.87±0.13	0.86±0.11	0.93±0.16	
A/G, Week 3	1.15±0.15	1.12±0.22	1.18±0.21	1.10±0.27	0.76±0.09	<0.001

As shown in Table 5, serum CREA content in Group 5 (Week 16) was significantly higher than in the control group and Groups 2 and 3 ($P<0.05$), while Groups 2 (Weeks 0 and 8), 3 (Week 0), 4 (Weeks 1 and 2), and 5 (Week 1) were significantly lower than the control group ($P<0.05$). For serum TBIL content, Group 5 (Week 1) was significantly higher than other groups ($P<0.05$), while other groups (Week 3) were significantly lower than the control group ($P<0.05$).

Table 5 Effects of *Bacillus subtilis* CGMCC 1.921 on serum CREA and TBIL contents of laying hens

Items	Group 1	Group 2	Group 3	Group 4	Group 5	P-value
CREA, Week 0	4.4±0.6	3.6±0.7	3.5±0.7	4.2±1.1	4.2±0.8	
CREA, Week 1	6.3±0.7	6.0±0.5	6.4±0.7	5.6±0.6	5.1±0.7	<0.001
CREA, Week 2	5.6±0.6	5.5±0.7	5.2±0.6	5.0±0.8	5.6±0.8	
CREA, Week 8	6.3±0.6	5.7±0.7	6.1±0.8	6.2±1.2	6.6±0.9	
CREA, Week 16	3.0±1.3	2.7±0.8	3.1±0.5	3.3±0.8	3.7±1.3	

Items	Group 1	Group 2	Group 3	Group 4	Group 5	P-value
CREA, Week 20	7.9±0.9	7.5±0.6	7.6±0.7	8.1±0.6	7.6±0.5	
TBIL, Week 1	2.1±0.5	2.3±0.7	2.2±0.6	2.1±0.7	2.8±0.5	<0.001
TBIL, Week 3	3.7±0.6	3.0±0.9	3.0±0.7	3.0±0.7	3.3±0.7	

As shown in Table 6, serum UREA content in Groups 2, 3, and 5 (Week 1) was significantly higher than in the control group ($P<0.05$), while Groups 2 (Weeks 8 and 12), 4 (Weeks 3 and 12), and 5 (Weeks 2, 3, and 12) were significantly lower than the control group ($P<0.05$). For serum UA content, Groups 2 and 5 (Week 20) were significantly lower than the control group and Group 3 ($P<0.05$).

Table 6 Effects of *Bacillus subtilis* CGMCC 1.921 on serum UREA and UA contents of laying hens

Items	Group 1	Group 2	Group 3	Group 4	Group 5	P-value
UREA (mmol/L), Week 1	0.28±0.07	0.33±0.07	0.34±0.07	0.30±0.08	0.33±0.06	
UREA (mmol/L), Week 2	0.36±0.06	0.35±0.05	0.35±0.07	0.35±0.07	0.29±0.05	
UREA (mmol/L), Week 3	0.38±0.06	0.34±0.05	0.35±0.07	0.31±0.04	0.33±0.09	
UREA (mmol/L), Week 8	0.38±0.13	0.30±0.10	0.38±0.08	0.39±0.07	0.40±0.08	
UREA (mmol/L), Week 12	0.50±0.07	0.44±0.09	0.50±0.09	0.40±0.07	0.41±0.12	<0.001
UA (mol/L), Week 20	204±77	146±40	206±79	181±73	158±81	

As shown in Table 7, serum GLU content in Groups 3 (Weeks 1 and 8), 4 (Week 8), and 5 (Weeks 1 and 8) was significantly lower than in the control group ($P<0.05$), while Groups 2 (Week 12), 3 (Weeks 2, 3, and 20), 4 (Weeks 3, 12, and 16), and 5 (Weeks 2, 3, 12, 16, and 20) were significantly higher than the control group ($P<0.05$).

Table 7 Effects of *Bacillus subtilis* CGMCC 1.921 on serum GLU content of laying hens, mmol/L

Items	Group 1	Group 2	Group 3	Group 4	Group 5	P-value
Week 0	11.09±1.52	10.59±1.44	12.07±2.00	11.97±1.64	11.48±1.50	
Week 1	12.15±0.99	11.80±1.12	11.10±0.99	11.79±1.25	11.20±1.06	
Week 2	7.89±1.22	7.46±1.28	8.99±1.12	8.56±0.82	9.94±1.45	<0.001
Week 3	7.40±0.96	7.14±0.99	8.18±1.15	8.17±1.15	8.86±1.11	<0.001
Week 4	9.37±1.44	9.33±1.16	10.08±1.49	9.50±1.43	10.00±1.56	
Week 8	10.41±0.97	10.11±0.97	8.97±1.29	8.78±1.12	9.32±1.05	<0.001
Week 12	7.57±1.10	9.19±1.57	8.53±1.06	9.69±1.49	10.35±2.56	<0.001
Week 16	7.84±1.78	8.58±1.64	8.49±1.13	9.30±2.83	9.68±1.63	
Week 20	9.23±0.81	9.42±1.38	10.45±3.14	9.73±0.93	11.15±0.98	
Week 24	13.53±0.95	13.47±1.11	13.57±1.48	13.29±0.73	12.98±1.24	

At other time points, there were no significant differences in serum ALT and AST activities or TP, ALB, GLB, TBIL, CREA, UREA, UA contents, and A/G ratio among groups (data not shown) ($P>0.05$). Dietary supplementation with *Bacillus subtilis* CGMCC 1.921 had no significant effect on serum CHOL and TG contents (data not shown) ($P>0.05$).

In summary, dietary supplementation with *Bacillus subtilis* CGMCC 1.921 increased serum GLU content and decreased serum UREA content to some extent, but had minimal effects on other serum biochemical parameters throughout the experimental period.

2.3 Effects of *Bacillus subtilis* CGMCC 1.921 on Hematological Parameters of Laying Hens

As shown in Table 8, blood WBC counts in Groups 2 (Weeks 1, 12, 16, and 24), 3 (Weeks 3, 16, 20, and 24), 4 (Weeks 1, 3, 4, 8, 16, 20, and 24), and 5 (Weeks 1, 3, 4, 8, 20, and 24) were significantly higher than in the control group ($P<0.05$). Group 2 (Week 12) was significantly higher than Groups 3 and 4 ($P<0.05$), Group 3 (Week 20) was significantly higher than Group 2 ($P<0.05$), Group 4 (Weeks 3 and 4) was significantly higher than Groups 2 and 3 ($P<0.05$), Group 4 (Weeks 8 and 20) was significantly higher than all other groups ($P<0.05$), Group 5 (Week 1) was significantly higher than all other groups ($P<0.05$), and Group 5 (Weeks 3, 4, and 20) was significantly higher than Group 2 ($P<0.05$).

Table 8 Effects of *Bacillus subtilis* CGMCC 1.921 on blood WBC count of laying hens, 10 /L

Items	Group 1	Group 2	Group 3	Group 4	Group 5	P-value
Week 0	121.1±32.7	118.1±12.2	129.5±22.3	114.2±23.5	128.3±28.7	
Week 1	117.1±7.1	130.0±9.6	126.4±4.5	128.2±13.1	140.0±32.6	<0.001
Week 2	125.5±10.4	122.9±5.6	133.0±22.6	123.2±6.9	124.4±12.6	
Week 3	118.6±6.2	119.2±9.9	122.7±3.8	127.1±3.9	125.4±7.5	<0.001
Week 4	120.3±9.4	121.4±16.6	127.9±4.2	138.2±23.4	131.8±15.5	<0.001
Week 8	124.6±7.9	129.0±13.9	128.1±3.8	139.1±15.7	132.1±8.5	<0.001
Week 12	127.4±3.8	140.3±22.1	126.4±12.0	124.5±16.9	133.0±7.0	
Week 16	124.8±6.5	133.4±11.1	133.8±7.8	137.5±21.6	130.9±9.0	
Week 20	124.6±5.4	123.1±6.8	131.8±5.4	138.2±13.0	131.5±8.3	<0.001
Week 24	122.8±5.2	130.0±24.1	131.0±3.7	134.4±10.4	136.4±7.3	

As shown in Table 9 , blood LYM counts in Groups 2 (Weeks 12, 16, and 24), 3 (Weeks 2, 3, 4, 20, and 24), 4 (Weeks 3, 4, 8, 20, and 24), and 5 (Weeks 3, 4, 8, 20, and 24) were significantly higher than in the control group ($P<0.05$). Group 4 (Weeks 0, 12, and 16) was significantly lower than all other groups ($P<0.05$), Group 2 (Week 4) was significantly lower than the control group ($P<0.05$), and Groups 3, 4, and 5 (Weeks 3, 4, and 8) were significantly higher than Group 2 ($P<0.05$).

Table 9 Effects of *Bacillus subtilis* CGMCC 1.921 on blood LYM count of laying hens, 10 /L

Items	Group 1	Group 2	Group 3	Group 4	Group 5	P-value
Week 0	102.6±18.4	110.6±9.8	112.0±14.7	85.0±22.9	105.5±6.9	<0.001
Week 1	105.9±4.5	113.2±7.1	111.9±13.2	106.0±20.5	109.3±16.6	
Week 2	108.0±6.9	108.1±5.3	118.5±7.6	106.2±4.7	103.0±14.3	<0.001
Week 3	107.0±3.8	105.7±7.0	112.5±3.2	117.8±4.3	110.9±8.3	<0.001
Week 4	105.9±8.5	90.0±23.7	114.0±2.8	119.8±15.3	115.3±9.8	<0.001
Week 8	108.9±7.2	104.2±28.0	114.8±3.4	122.2±9.2	117.8±6.8	<0.001
Week 12	115.8±2.9	122.5±10.6	112.1±10.3	104.1±9.9	114.6±4.2	<0.001
Week 16	114.6±2.8	121.5±5.3	112.3±6.0	104.9±14.3	109.9±7.3	<0.001
Week 20	104.6±1.9	107.7±4.9	117.7±2.8	114.4±10.9	109.2±3.1	<0.001
Week 24	104.6±1.9	108.0±5.6	116.3±2.1	109.8±7.8	109.7±2.6	<0.001

As shown in Table 10 , blood MID counts in Groups 2 (Weeks 1 and 12), 3 (Week 16), 4 (Weeks 0, 12, 16, 20, and 24), and 5 (Weeks 0, 1, 3, 12, 16, 20, and 24) were significantly higher than in the control group ($P<0.05$). Groups 4 and 5 (Weeks 0, 20, and 24) were significantly higher than all other groups ($P<0.05$), and Group 5 (Weeks 1 and 3) was significantly higher than Groups 3 and 4 ($P<0.05$).

Table 10 Effects of *Bacillus subtilis* CGMCC 1.921 on blood MID count of laying hens, 10 /L

Items	Group 1	Group 2	Group 3	Group 4	Group 5	P-value
Week 0	7.7±2.4	5.9±2.3	6.4±3.9	11.3±4.3	13.8±8.8	<0.001
Week 1	8.4±2.2	11.5±2.1	9.2±2.2	9.3±4.6	12.5±7.1	
Week 2	11.3±3.4	10.2±2.3	8.9±6.4	12.2±3.7	12.6±3.7	
Week 3	8.6±2.2	9.3±2.8	8.0±1.6	7.0±1.9	10.8±4.0	<0.001
Week 4	10.4±2.0	12.7±4.9	10.0±1.8	10.4±6.5	11.4±5.9	
Week 8	11.2±2.1	12.2±2.3	9.8±1.9	10.6±4.5	10.1±3.8	
Week 12	8.6±1.3	11.8±5.7	10.3±2.1	12.2±4.4	12.2±2.8	<0.001
Week 16	7.5±2.8	8.5±3.4	15.2±3.7	16.8±6.0	14.9±3.2	<0.001
Week 20	13.5±2.8	10.7±2.2	10.5±2.1	16.1±5.9	15.7±4.3	<0.001
Week 24	12.6±2.4	12.9±6.2	10.5±1.7	16.0±7.4	18.3±2.6	<0.001

As shown in Table 11 , blood NEU counts in Groups 2 (Week 1), 3 (Week 16), 4 (Weeks 0, 12, 16, and 24), and 5 (Weeks 0, 12, 16, and 24) were significantly higher than in the control group (P<0.05). Group 3 (Weeks 0, 2, and 20) was significantly lower than the control group (P<0.05), and Groups 4 and 5 (Weeks 0, 12, and 24) were significantly higher than Groups 2 and 3 (P<0.05).

Table 11 Effects of *Bacillus subtilis* CGMCC 1.921 on blood NEU count of laying hens, 10 /L

Items	Group 1	Group 2	Group 3	Group 4	Group 5	P-value
Week 0	2.5±1.6	1.6±1.1	1.1±0.3	4.3±2.2	3.4±1.3	<0.001
Week 1	2.8±1.1	5.3±1.4	3.1±1.5	2.1±1.2	2.6±1.3	<0.001
Week 2	4.4±3.2	4.6±2.0	1.7±0.8	4.8±1.3	4.1±1.5	<0.001
Week 3	3.1±1.8	4.2±2.7	2.2±0.6	2.3±2.0	3.8±2.2	
Week 4	4.1±1.4	4.8±2.2	3.9±1.8	3.8±3.0	4.0±3.0	
Week 8	4.5±2.3	5.3±2.3	3.5±1.0	3.5±2.8	4.2±2.8	
Week 12	3.0±0.9	4.2±2.7	3.9±2.1	6.9±4.4	6.2±2.4	<0.001
Week 16	2.6±2.1	2.6±1.8	6.3±3.3	6.6±5.6	6.1±2.5	<0.001
Week 20	6.5±2.4	4.7±2.2	3.6±1.1	7.7±4.3	6.5±4.2	<0.001
Week 24	5.5±1.8	4.8±3.0	4.2±1.3	8.5±5.7	8.4±4.0	<0.001

As shown in Table 12 , blood RBC counts in Groups 2 (Weeks 1 and 3), 4 (Weeks 2 and 12), and 5 (Weeks 1, 2, 3, and 12) were significantly lower than in the control group (P<0.05), while Groups 3, 4, and 5 (Weeks 16 and 20) were significantly higher than the control group and Group 2 (P<0.05).

Table 12 Effects of *Bacillus subtilis* CGMCC 1.921 on blood RBC count of laying hens, 10¹²/L

Items	Group 1	Group 2	Group 3	Group 4	Group 5	P-value
Week 0	2.19±0.23	2.11±0.31	2.15±0.18	2.13±0.29	2.06±0.25	
Week 1	2.42±0.20	2.28±0.18	2.40±0.19	2.33±0.13	2.20±0.25	<0.001
Week 2	2.35±0.13	2.39±0.18	2.31±0.14	2.23±0.22	2.20±0.13	<0.001
Week 3	2.43±0.15	2.24±0.30	2.39±0.17	2.35±0.13	2.30±0.18	
Week 4	2.26±0.14	2.29±0.17	2.30±0.16	2.35±0.19	2.30±0.14	
Week 8	2.32±0.12	2.40±0.15	2.42±0.16	2.37±0.18	2.32±0.25	
Week 12	2.45±0.15	2.53±0.17	2.42±0.21	2.32±0.18	2.28±0.20	<0.001
Week 16	2.42±0.17	2.43±0.22	2.58±0.21	2.61±0.22	2.60±0.29	
Week 20	2.38±0.17	2.38±0.16	2.52±0.15	2.56±0.20	2.56±0.30	
Week 24	2.45±0.15	2.55±0.20	2.47±0.19	2.57±0.18	2.56±0.13	

As shown in Table 13 , blood HGB content in Groups 2 (Weeks 1, 3, 4, and 20), 3 (Weeks 3, 20, and 24), 4 (Weeks 1, 3, and 12), and 5 (Weeks 1 and 12) was significantly lower than in the control group ($P<0.05$), while Groups 3, 4, and 5 (Week 16) were significantly higher than the control group and Group 2 ($P<0.05$).

Table 13 Effects of *Bacillus subtilis* CGMCC 1.921 on blood HGB content of laying hens, g/L

Items	Group 1	Group 2	Group 3	Group 4	Group 5	P-value
Week 0	91±11	86±15	93±10	90±8	91±11	
Week 1	103±9	91±8	98±9	96±6	93±10	<0.001
Week 2	101±7	96±12	100±7	98±7	101±7	
Week 3	104±7	93±14	98±7	95±7	98±10	
Week 4	98±7	90±12	95±7	97±9	98±7	
Week 8	101±6	96±12	101±6	100±7	101±6	
Week 12	103±8	106±7	105±7	97±7	94±8	<0.001
Week 16	102±9	101±8	110±8	109±9	108±9	<0.001
Week 20	110±8	104±9	103±6	106±8	113±11	
Week 24	110±8	111±8	104±8	110±8	112±7	

As shown in Table 14 , blood HCT in Groups 2 (Week 3), 4 (Weeks 2, 3, and 12), and 5 (Weeks 1, 2, 3, and 12) was significantly lower than in the control group ($P<0.05$), while Groups 2 (Week 24), 3 (Weeks 16, 20, and 24), 4 (Weeks 16, 20, and 24), and 5 (Weeks 16, 20, and 24) were significantly higher than the control group ($P<0.05$).

Table 14 Effects of *Bacillus subtilis* CGMCC 1.921 on HCT in blood of laying hens, %

Items	Group 1	Group 2	Group 3	Group 4	Group 5	P-value
Week 0	26.8±3.0	25.1±4.0	26.0±2.2	25.6±2.3	25.2±3.1	
Week 1	29.3±2.4	28.3±2.3	29.1±2.1	28.0±1.6	26.2±3.0	<0.001
Week 2	28.4±1.6	28.9±2.2	27.5±1.9	27.0±2.3	26.6±1.8	<0.001
Week 3	29.1±1.9	26.8±3.5	28.1±1.9	27.4±1.9	27.6±2.3	
Week 4	27.6±1.9	27.4±2.1	28.1±1.8	28.5±2.1	28.1±1.5	
Week 8	28.3±1.6	29.0±1.6	29.5±1.8	29.0±2.3	28.6±3.0	
Week 12	29.5±2.1	30.4±2.1	29.0±2.1	27.6±2.1	27.6±2.4	<0.001
Week 16	29.1±2.4	28.8±2.5	30.6±2.2	30.9±2.5	31.2±3.1	
Week 20	27.7±2.0	27.7±2.2	30.7±1.8	30.4±2.2	30.8±3.4	<0.001
Week 24	28.6±1.9	29.9±2.6	30.0±2.3	30.5±1.8	30.5±1.6	

As shown in Table 15, blood PLT counts in Groups 2 (Week 24), 3 (Week 24), 4 (Weeks 16 and 24), and 5 (Weeks 16 and 24) were significantly higher than in the control group ($P < 0.05$).

Table 15 Effects of *Bacillus subtilis* CGMCC 1.921 on blood PLT count of laying hens, $10^9/L$

Items	Group 1	Group 2	Group 3	Group 4	Group 5	P-value
Week 0	11.4±2.6	13.9±2.6	12.0±4.5	12.9±3.7	14.0±4.5	
Week 1	12.7±9.2	13.6±2.6	10.9±4.5	12.9±3.6	12.9±3.6	
Week 2	9.3±3.7	8.9±4.4	12.5±4.0	11.5±6.5	10.4±5.9	
Week 3	10.7±3.4	12.6±5.3	11.2±4.0	13.4±3.2	13.0±2.8	
Week 4	10.1±3.8	11.0±5.4	10.3±4.6	10.7±6.2	10.3±5.0	
Week 8	12.0±5.7	11.0±4.1	11.2±3.9	12.0±5.4	12.3±3.4	
Week 12	11.6±5.4	11.5±5.6	9.5±5.4	13.6±2.6	13.0±4.7	
Week 16	12.3±5.8	11.8±4.9	16.3±9.1	20.1±10.3	19.1±9.0	
Week 20	9.4±4.5	9.3±5.9	12.0±5.0	11.6±5.9	10.6±6.6	
Week 24	7.5±4.2	10.3±5.3	14.2±3.2	12.8±4.2	15.3±5.3	<0.001

As shown in Table 16, blood ESR in Groups 2 (Week 20), 3 (Weeks 3, 20, and 24), 4 (Weeks 3 and 20), and 5 (Weeks 3, 4, 8, and 20) was significantly higher than in the control group ($P < 0.05$).

Table 16 Effects of *Bacillus subtilis* CGMCC 1.921 on ESR in blood of laying hens, mm/h

Items	Group 1	Group 2	Group 3	Group 4	Group 5	P-value
Week 0	1.8±0.7	2.3±0.9	2.1±0.4	1.8±0.7	2.0±0.8	

Items	Group 1	Group 2	Group 3	Group 4	Group 5	P-value
Week 1	2.0±0.0	2.0±0.2	2.0±0.5	2.2±0.7	2.0±0.4	
Week 2	2.0±0.4	1.7±0.6	1.8±0.4	2.0±0.6	2.3±0.7	
Week 3	1.3±0.5	1.5±0.5	1.9±0.4	2.3±1.1	1.7±0.6	<0.001
Week 4	1.5±0.6	1.7±0.6	1.7±0.5	1.7±0.6	2.2±0.5	
Week 8	1.6±0.6	1.8±0.4	1.5±0.5	1.8±0.4	2.0±0.0	
Week 12	1.9±0.3	2.0±0.4	2.2±0.9	2.2±0.9	1.9±0.3	
Week 16	1.9±0.4	2.0±0.6	1.6±0.5	1.8±0.5	1.8±0.4	
Week 20	1.0±0.0	1.5±0.6	1.5±0.6	1.7±0.7	1.8±0.5	
Week 24	1.2±0.5	1.4±0.5	1.7±0.5	1.4±0.5	1.5±0.5	

In summary, dietary supplementation with *Bacillus subtilis* CGMCC 1.921 increased blood WBC and LYM counts, decreased blood RBC counts and HCT during Weeks 1, 2, 3, and 12, but increased blood RBC and PLT counts and HCT during Weeks 16, 20, and 24.

2.4 Effects of *Bacillus subtilis* CGMCC 1.921 on Serum Immunoglobulin Contents of Laying Hens

As shown in Table 17, dietary supplementation with *Bacillus subtilis* CGMCC 1.921 tended to increase serum IgA content (Week 3) ($P=0.083$). For serum IgG content, Group 5 (Week 1) was significantly higher than the control group and Groups 2 and 3 ($P<0.05$), Groups 4 and 5 (Week 4) were significantly higher than the control group ($P<0.05$), while Group 2 (Week 20) was significantly lower than the control group and Group 3 ($P<0.05$). For serum IgM content, Group 5 (Weeks 2 and 8) was significantly higher than all other groups ($P<0.05$), Groups 3, 4, and 5 (Week 4) were significantly higher than the control group ($P<0.05$), and Group 3 (Week 16) was significantly higher than all other groups ($P<0.05$). At other time points, there were no significant differences in serum IgA, IgG, and IgM contents among groups (data not shown) ($P>0.05$).

Table 17 Effects of *Bacillus subtilis* CGMCC 1.921 on serum immunoglobulin contents of laying hens, mg/mL

Items	Group 1	Group 2	Group 3	Group 4	Group 5	P-value
IgA, Week 3	1.96±0.32	2.03±0.30	2.08±0.32	2.22±0.48	2.74±0.85	
IgG, Week 1	2.90±1.11	2.34±0.70	2.90±1.03	3.54±0.90	4.25±1.17	
IgG, Week 4	1.89±0.53	2.09±0.60	2.67±0.44	2.88±0.59	3.56±0.95	

Items	Group 1	Group 2	Group 3	Group 4	Group 5	P-value
IgG, Week 16	3.91±1.14	5.57±1.16	4.77±1.34	4.62±1.43	3.62±1.11	
IgG, Week 20	4.90±0.67	3.59±0.63	5.09±0.98	4.58±1.17	4.24±0.64	
IgM, Week 2	1.51±0.31	1.40±0.25	1.44±0.16	1.56±0.33	2.25±0.26	<0.001
IgM, Week 4	0.97±0.35	1.13±0.23	1.50±0.30	1.45±0.48	2.47±0.39	<0.001
IgM, Week 8	2.21±0.22	2.19±0.20	2.27±0.23	2.53±0.43	2.97±0.29	<0.001
IgM, Week 16	2.72±0.63	2.87±0.93	4.19±0.37	2.76±1.26	3.18±0.77	

3.1 Effects of *Bacillus subtilis* CGMCC 1.921 on Performance of Laying Hens

Studies have shown that dietary probiotic supplementation can improve animal performance, though conclusions vary. This study found that compared with the control group, supplementation with 1×10^8 , 1×10^7 , 1×10^6 , and 1×10^5 CFU/g *Bacillus subtilis* CGMCC 1.921 had no significant effect on egg production rate, but increased it by 3.24%, 3.35%, 3.35%, and 4.02%, respectively. Supplementation with 1×10^8 , 1×10^7 , and 1×10^6 CFU/g *Bacillus subtilis* CGMCC 1.921 significantly reduced the feed-to-egg ratio, with no significant differences among the four supplementation levels. Therefore, under the conditions of this experiment, the appropriate supplementation level of *Bacillus subtilis* CGMCC 1.921 was 1×10^6 CFU/g. Zhang et al. [11] reported that dietary supplementation with 3.6×10^8 CFU/g *Bacillus subtilis* significantly reduced the feed-to-egg ratio without significantly affecting egg production rate, egg weight, or feed intake, but increased egg production rate to some extent, which is consistent with our results. Ribeiro et al. [12] found that dietary supplementation with 8×10^8 CFU/g *Bacillus subtilis* could increase egg production rate and egg weight. Additionally, studies have shown that dietary supplementation with *Bacillus subtilis* and *Lactobacillus plantarum* can improve growth performance in broilers [13], piglets [14], and aquatic animals [15]. Deng et al. [16] found that dietary supplementation with 1×10^8 CFU/g *Bacillus licheniformis* could alleviate the adverse effects of heat stress on laying hen performance. These effects may be related to probiotics' ability to enhance digestive enzyme activity [17], improve intestinal structure [18-19], and increase nutrient utilization [20]. However, Forte et al. [21] found that supplementation with 0.1% *Lactobacillus acidophilus* and 0.05% *Bacillus subtilis* had no significant effects on the performance of Hy-Line laying hens. Sobczak et al. [22] reported that *Bacillus subtilis* ATCC PTA-6737 did not improve the performance of Lohmann Brown laying hens. These discrepancies may be related to supplementation level, animal production stage,

rearing environment, or strain characteristics.

3.2 Effects of *Bacillus subtilis* CGMCC 1.921 on Serum Biochemical Parameters of Laying Hens

This experiment found that dietary supplementation with different levels of *Bacillus subtilis* CGMCC 1.921 had essentially no effect on serum ALT and AST activities during the experimental period. ALT and AST are primarily found in liver cells, and their activity reflects the degree of liver cell damage. When liver cells are damaged, large amounts of ALT and AST are released into the blood, increasing serum activity. Some studies have shown that dietary probiotic supplementation can significantly reduce serum ALT and AST activities [21,23]. However, Li et al. [24] and Ma et al. [25] reported that dietary *Bacillus subtilis* supplementation had no significant effect on serum ALT and AST activities in Shaoxing ducks and laying hens, consistent with our results. Serum TP, ALB, GLB contents, and A/G ratio are indicators of liver synthesis and reserve function, while serum TBIL content reflects normal liver secretion and excretion function. ALB is synthesized by the liver; liver damage reduces serum ALB content, while liver inflammation increases serum GLB and TBIL contents. There were no major differences in serum TP, ALB, GLB, TBIL contents, and A/G ratio between experimental and control groups, indicating that dietary supplementation with different levels of *Bacillus subtilis* CGMCC 1.921 did not adversely affect liver function in laying hens. UREA, UA, and CREA are important indicators of kidney function. This experiment showed that *Bacillus subtilis* CGMCC 1.921 supplementation had no significant improving effect on serum UA and CREA contents but reduced serum UREA content. UREA is a product of protein metabolism that reflects the level of protein catabolism. Generally, increased serum UREA content indicates decreased protein deposition. The reduced serum UREA content in this experiment suggests that *Bacillus subtilis* CGMCC 1.921 supplementation improved protein deposition and reduced UREA accumulation in the kidneys, thereby providing some kidney protection. Hatab et al. [26] reported that dietary supplementation with *Bacillus subtilis* and *Enterococcus faecium* significantly reduced serum UA content in laying hens without significantly affecting serum CREA content, and significantly increased serum GLU content. This experiment also found that *Bacillus subtilis* CGMCC 1.921 supplementation increased serum GLU content to some extent. GLU is the main energy source for the body, and its increased content indicates that *Bacillus subtilis* CGMCC 1.921 supplementation can provide relatively more energy to the organism. The increased serum GLU content may be due to enhanced carbohydrate digestion, glycogenolysis, and gluconeogenesis resulting from probiotic supplementation. Regarding serum CHOL and TG contents, Forte et al. [21] and Hatab et al. [26] reported that *Bacillus subtilis* and *Enterococcus faecium* supplementation could reduce serum CHOL and TG contents in laying hens. This experiment found that different levels of *Bacillus subtilis* CGMCC 1.921 supplementation had no effect on serum CHOL and TG contents at any experimental stage. Sobczak et al. [22] reported that dietary

Bacillus subtilis ATCC PTA-6737 had no significant effect on serum CHOL and TG contents in laying hens, consistent with our results. These differences may be related to experimental duration, bacterial species, or strain characteristics.

3.3 Effects of *Bacillus subtilis* CGMCC 1.921 on Hematological Parameters and Immunoglobulin Contents of Laying Hens

Whole blood hematological parameters reflect the health status of the organism. WBCs are important blood cells that defend against pathogens. LYMs reflect the immune function level and play a major role in the immune response. MID includes monocytes, eosinophils, and basophils. Monocytes and NEUs have strong phagocytic functions and play important roles in the non-specific cellular immune system. Studies have shown that probiotics can prevent disease, control infection, and enhance immunity [27]. Khan et al. [28] found that multi-strain probiotics significantly increased LYM percentage in laying hens. This study showed that dietary *Bacillus subtilis* CGMCC 1.921 supplementation increased blood WBC and LYM counts, with more pronounced effects at supplementation levels of 1×10^8 and 1×10^9 CFU/g. Kim et al. [29] also reported that dietary probiotic supplementation (*Bacillus subtilis*, lactic acid bacteria, and yeast) significantly increased blood WBC and LYM counts in laying hens, similar to our results. This suggests that probiotics or their metabolites may stimulate the organism and exert certain immunomodulatory effects. Park et al. [30] fed broilers challenged with *Salmonella typhimurium* KCCM 40253 diets containing *Bacillus subtilis* RX7 and B2A for 10 days and found no significant effects on blood WBC and LYM counts. RBCs are the most numerous blood cells and serve as the primary medium for oxygen transport via blood, in addition to having immune functions. Gheisar et al. [31] found that dietary *Enterococcus faecium* supplementation had no significant effect on blood RBC counts in broilers. However, Stropfova et al. [32] reported that dietary *Enterococcus faecium* supplementation significantly increased blood RBC counts and HCT in piglets. Bonsu et al. [33] found that multi-strain probiotic supplementation in broilers had no effect on blood RBC counts but reduced blood HGB content. This study showed that dietary *Bacillus subtilis* CGMCC 1.921 supplementation reduced blood HGB content and RBC counts and HCT during the first 12 weeks, but significantly increased blood RBC counts and HCT during Weeks 16, 20, and 24. These changes may be related to the physiological status of the animals, though the specific mechanisms require further investigation.

Regarding blood PLT counts, Elzey et al. [34] reported that PLTs play important roles in the immune system, and reduced PLT counts in normal mice lead to decreased specific antibody production. This experiment showed that compared with the control group, Group 5 significantly increased blood PLT counts during Weeks 16 and 20. Telli et al. [35] found that *Bacillus subtilis* supplementation in Nile tilapia raised at high stocking density had no significant effect on blood PLT counts, but significantly increased them at low stocking density. Regarding blood ESR, this experiment showed a slight increase in blood ESR

to some extent. Increased blood ESR may be related to decreased blood RBC counts or increased serum GLU content, but other hematological parameters showed no obvious adverse changes, indicating that the changes in blood ESR did not affect the organism. Furthermore, dietary *Bacillus subtilis* CGMCC 1.921 supplementation could increase serum immunoglobulin content to some extent, particularly significantly increasing serum IgM content, with the best effect observed at a supplementation level of 1×10^8 CFU/g under the conditions of this experiment. This indicates that *Bacillus subtilis* CGMCC 1.921 can enhance the immune defense mechanism to a certain extent. Kim et al. [29] reported that feeding laying hens with lactic acid bacteria probiotics significantly increased serum IgG content, while showing a non-significant increase in broilers. Choi et al. [36] found that feeding weaned piglets a probiotic preparation containing *Bacillus subtilis*, *Lactobacillus acidophilus*, and *Saccharomyces cerevisiae* had no significant effect on serum IgA, IgG, and IgM contents. Bao et al. [37] reported that supplementation with 1×10^8 , 1×10^7 , 1×10^6 , and 1×10^5 CFU/g *Lactobacillus johnsonii* significantly reduced serum IgA content on days 7 and 35, but significantly increased it on day 28.

Conclusions

1. Dietary supplementation with *Bacillus subtilis* CGMCC 1.921 can reduce the feed-to-egg ratio of laying hens, with an appropriate supplementation level of 1.0×10^8 CFU/g.
2. Dietary supplementation with *Bacillus subtilis* CGMCC 1.921 can increase serum GLU content and reduce serum UREA content to some extent, but has no significant improving effect on other serum biochemical parameters.
3. Dietary supplementation with *Bacillus subtilis* CGMCC 1.921 can increase blood WBC and LYM counts and serum immunoglobulin content, thereby enhancing the immune function of laying hens to a certain extent, with an appropriate supplementation level of 1.0×10^8 CFU/g.

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