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Effects of Dietary *Bacillus licheniformis* on Gut Physical Barrier, Immunity, and Reproductive Hormones of Laying Hens

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Abstract Previous study showed that dietary *Bacillus licheniformis* (*B. licheniformis*) administration contributes to the improvement of laying performance and egg quality in laying hens. In this study, we aimed to further evaluate its underlying mechanisms. Three hundred sixty Hy-Line Variety W-36 hens (28 weeks of age) were randomized into four groups, each group with six replications (n = 15). The control group received the basal diet and the treatment groups received the same basal diets supplemented with 0.01, 0.03, and 0.06% *B. licheniformis* powder (2×10^{10} cfu/g) for an 8-week trial. The results demonstrate that *B. licheniformis* significantly enhance the intestinal barrier functions via decreasing gut permeability, promoting *mucin-2* transcription, and regulating inflammatory cytokines. The systemic immunity of layers in *B. licheniformis* treatment groups is improved through modulating the specific and non-specific immunity. In addition, gene expressions of hormone receptors, including estrogen receptor α, estrogen receptor β, and follicle-stimulating hormone receptor, are also regulated by *B. licheniformis*. Meanwhile, compared with the control, *B. licheniformis* significantly increase gonadotropin-releasing hormone level, but markedly reduce ghrelin and inhibin secretions. Overall, our data suggest that dietary inclusion of *B. licheniformis* can improve the intestinal barrier function and systemic immunity and regulate reproductive hormone secretions, which contribute to better laying performance and egg quality of hens.

Keywords *Bacillus licheniformis* · Intestinal barrier function · Hormone · Immunity

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Introduction

Inclusion of probiotics in diets for layers is preferred to replace antibiotics [1] and improve growth performance, feed conversion efficiency, and egg quality [2–4]. It is reported that probiotics have beneficial impacts on the poultry performance by synthesizing vitamins [5], inducing the digestive enzyme [6], utilizing undigestible carbohydrates [7], stimulating lactic acid, and releasing bacteriocins [8]. Several probiotics, such as *Lactobacillus*, *Streptococcus*, *Saccharomyces*, *Aspergillus*, and *Bacillus*, have been selected and applied in poultry production [9]. However, *Bacillus*, the spore-forming bacteria, are ideally suited as feed additives, because they have higher resistance to harsh environments [10] and have the ability to produce a variety of enzymes including protease, amylase, and lipase [11]. *Bacillus licheniformis*, which has been broadly applied in livestock and aquaculture as growth promoter and competitive exclusion agent [12, 13], has demonstrated a positive effect in aiding nutrient digestion and absorption in the host's body [13, 14], inhibiting the growth and reproduction of pathogens by producing antimicrobial active substances and reacting with oxygen that retards the growth and reproduction of pathogens [15].

In our previous study, we found that dietary *B. licheniformis* supplementation effectively improves laying performance and egg quality by increasing eggshell thickness and strength in a dose-dependent manner in laying hens via decreasing the stress response, up-regulating the growth

hormones, and improving intestinal mucosal structure [16]. In recent years, it has been reported that probiotics also benefit animals by enhancing intestinal barrier function and positively modulating the immune system [17]. However, to our knowledge, little research has been conducted on related mechanisms of *B. licheniformis* in improving laying performance and egg quality in layers in the respect of immunity and gut health. The objective of this study is to further explore the effects of dietary *B. licheniformis* supplementation on laying hens and its molecular mechanisms in terms of gut barrier, the systemic immunity, and hormone gene expression.

Materials and Methods

Birds and Management

The experiment was carried out in accordance with the Chinese guidelines for animal welfare and approved by the Animal Welfare Committee of Animal Science College, Zhejiang University. The birds and management is referred to Lei et al. [16]. Briefly, a total of 360 Hy-Line Variety W-36 hens, 28 weeks of age, were randomly divided into four groups, each group with six replications and each replication with 15 hens.

Bacterial Strain and Diet

B. licheniformis strain was obtained from China General Microbiological Culture Collection Center (CGMCC1.3448) and prepared by Microbiology and Genetic Engineering Laboratory, Institute of Feed Sciences, Zhejiang University, China. *B. licheniformis* was cultured in Luria-Bertani media, kept at 37 °C for 24 h, and shaken at 180 r/min. Bacteria suspensions were centrifuged to obtain pure bacterial cells at 5000 g for 10 min at 4 °C. Subsequently, bacteria were washed twice with sterile 0.85% (8.5 g/L) sodium chloride solution. The culture purity and identification were constantly checked by the spreading plate method [18]. *B. licheniformis* powders (2×10^{10} cfu/g) were added to the basal diet at levels of 0.01, 0.03, and 0.06% to form the three types of treatment diets. Starch was used to dilute *B. licheniformis*, and the same amount of starch was also added to each group to compensate for the difference in nutrient composition of the diets. *B. licheniformis* powders were stored at room temperature. The composition and nutrition of the basal experimental diet can be found in our previous study [16]. Diets were stored in a dry and well-ventilated storeroom.

Sample Collection

At the end of the experiment, no hens died. Birds were fasted for 12 h [19, 20] and blood samples of 12 hens (two birds per replicate) were drawn from the axillary vein into vacuum tubes (5 mL) containing coagulant. After centrifugation for 10 min at 4 °C (3000×*g*, Centrifuge 5804R, Eppendorf, Germany), pure serum samples were obtained. The ovaries and mid-jejunum segments were carefully dissected and rinsed with sterilized saline. Jejunal mucosa was gently scraped off. All the samples were placed in liquid nitrogen immediately and stored at −70 °C till further analysis.

RNA Extraction and RT-qPCR

The RNA extraction and RT-qPCR were referred to previous research [21]. Briefly, total RNA was extracted using RNAiso Plus method (TaKaRa, Dalian, China). Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using M-MLV reverse transcriptase (TaKaRa, Dalian, China). Transcriptional changes were then identified by quantitative PCR, which was performed using the Premix Ex TaqTM with SYBR Green (TaKaRa, Dalian, China) and the ABI 7500 Fast Real-Time PCR system (Applied Biosystemics, Carlsbad, CA, USA). The thermocycle protocol lasted for 30 s at 95 °C, followed by 40 cycles of 5-s denaturation at 95 °C, 34-s annealing/extension at 60 °C, and then a final melting curve analysis to monitor purity of the PCR product. Primer sequences are presented in Table 1. The $2^{-\Delta\Delta Ct}$ method was used to estimate mRNA abundance. ΔC_t is $C_{t,\ \mathrm{target}} - C_{t,\ \mathrm{reference}}$ and $\Delta\Delta C_t$ is $\Delta C_{t,\ \mathrm{treatment}} - \Delta C_{t,\ \mathrm{control}}$ [21]. Relative gene expression levels were normalized to those of the eukaryotic reference gene *β-actin*.

ELISA

Levels of immunoglobulin A (IgA), immunoglobulin G (IgG), interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-10, and tumor necrosis factor α (TNF-α) were quantified using a sandwich ELISA kit (Komabiotech Ltd., Seoul, Korea) according to instructions. Briefly, serum samples were pipetted into wells coated with antibodies specific for IgA, IgG, IL-1β, IL-2, IL-4, IL-6, IL-10, and TNF-α. After incubation, biotinylated monoclonal secondary antibiotics were added, followed by streptavidin-peroxidase. After incubation and washing, the bound cytokines were visualized by developing the peroxidase reaction through the addition of H_2O_2 and the absorbency of each well was determined by SpectraMax M5 (MD, USA) [22].

Statistical Analysis

Data were statistically analyzed by one-way ANOVA procedure of SPSS 16.0 for Windows (SPSS Inc., Chicago, IL). When significant differences were found ($P < 0.05$), Tukey's test was further performed. Statements of significance were

Table 1 Gene name, primer sequences

Gene	Primer sequence
β-actin	F:5′GAGAAATTGTGCGTGACATCA3′ R:5′CCTGAACCTCTCATTGCCA3′
Cldn-1	F:5′TACTTTCCTGCTCCTGTCC3′ R:5′AAGGCGTTAATGTCAATCC3′
Cldn-2	F:5′CAAGGACCGAGTGGCAGTG3′ F: 5′TTTGATGGAGGGCTGAGGA3′
ZO-1	F:5′GCCTCCTGAGTTTGATAGTGG3′ R:5′CTCGGCAGACCTTGAAATAGA3′
OCLN-1	F:5′AATGCTTTCTCAGCCAGCGTAT3′ R:5′GCAAGCGTGGAGGCAACA3′
MUC2	F:5′ACGACTTTGACGGACACTGCT3′ R:5′AGGGGACGTTCTCGGTGAT3′
TNF-α	F:5′CTCCGCAGTACTCAGGACAGC3′ R:5′TCAGAGCATCAACGCAAAAGG3′
IL-1	F:5′TTCCGCTACACCCGCTCACA3′ R:5′TGCCGCTCATCACACACGAC3′
IL-4	F:5′TGATCTTTGGCTGTATTTCGG3′ R:5′ACTCCTGGGTCTCAGTTGGGG3′
IL-6	F:5′ATGAACTTCACCGAGGGCTGCGAGG3′ R:5′CCTCGCAGCCCTCGGTGAAGTTCAT3′
IL-10	R:5′ACTTAACATCCAACTGCTCAGC3′ R:5′CATCCATCTTCTCGAACGTCTC3′
ESRα	F:5′CTCTCACCCTTCATCCATCACC′3 R:5′CCTCACAAGACCAGACCCCATA3′
ESRβ	F:5′AAGAAGAGAACGCTGTGGGTAT3′ R:5′CTCGGTGAATGGTTTGCTAGGA3′
FSHR	F:5′CCAATGCCACAGAACTGAGATT3′ R:5′CTTATGGACGACGGGTAAAAAG3′
GnRH	F:5′TGCTTGGCTCAACACTGGTCTT3′ R:5′TCCTTTCTTCTGGCTTCTCCTT3′
Ghrelin	F:5′GCTCTGGCTGGCTCTAGTTTTT3′ R:5′TTCTGTGCCTCGGCGATGTAAT3′
INH	F:5′CAAAAGGATGTGAGGAGGGTGC3′ R:5′CCGAGGGCTGGAAGAGGTAAGT3′

F forward, *R* reverse

based on $P < 0.05$. The data were expressed as the means ± SEM.

Results

The Transcript Level of Genes Related to Intestinal Physical Barrier Function

We previously demonstrated that *B. licheniformis* lead to the improvement of intestinal mucosa structure [16]. In the presence of an intact epithelial cell layer, the paracellular pathway between cells must be sealed. This function is achieved by physical barrier, especially by tight junctions [23]. Besides, mucin 2 (MUC2) is the most abundant mucin, which creates the first line of defense against microbial encroachment [24]. Hence, here, we focused on the transcriptions of genes encoding tight junctions and mucin (MUC) in jejunal mucosa to further explore the physical barriers of layers. Figure 1a shows that *B. licheniformis* at three dosages all significantly increase *claudin 1* (CLDN-1) gene expressions, while only 0.03% *B. licheniformis* up-regulate the *claudin 2* (CLDN-2) levels. Although 0.01 and 0.03% *B. licheniformis* have no obvious effects on *zonula occludens 1* (ZO-1) and *occluding 1* (OCLIN-1), 0.06% *B. licheniformis* markedly elevate their expressions. In addition, *B. licheniformis* at three dosages dramatically up-regulate MUC2 expressions as well (Fig. 1b).

The Transcript Level of Genes Related to the Intestinal Immunological Barrier Function

Compared to the control group, the IL-6 and TNF-α transcriptions in jejunal mucosa of 0.01, 0.03, and 0.06% *B. licheniformis* groups show no significant changes. While, IL-1β expression in 0.03% *B. licheniformis* group is significantly decreased and this is reversed to normal when the *B. licheniformis* dosage reaches to 0.06% (Fig. 2a). Moreover, IL-10 and IL-4 mRNA expressions of all the *B. licheniformis* treatments are also elevated, but 0.01% *B. licheniformis* do not alter the IL-4 transcript level significantly (Fig. 2b).

The Transcript Level of Genes Encoding Hormones and Hormone Receptors

Our previous findings indicate that *B. licheniformis* at three dosages can significantly enhance levels of estradiol (E2) and follicle-stimulating hormone (FSH) [16]. We therefore investigated the mRNA expression of genes encoding estrogen receptor (ESR) and FSH receptor (FSHR) to further verify the impact of *B. licheniformis* on hormone secretions. Figure 3a shows that 0.01 and 0.03% *B. licheniformis* dramatically up-regulate ESRα and ESRβ expressions respectively, while 0.06% *B. licheniformis* decrease FSHR transcript levels markedly. Furthermore, *B. licheniformis* of three concentrations significantly increase gonadotropin-releasing hormone (GnRH) expressions and decrease inhibin (INH) transcriptions compared with the control group, while 0.03 and 0.06% *B. licheniformis* down-regulate *Ghrelin* levels significantly in a dose-dependent manner (Fig. 3b).

The Systemic Immunity

The effects of *B. licheniformis* on immunoglobulin and cytokine levels in serum are also evaluated. Results reveal that compared to the control group, 0.01% *B. licheniformis* have no obvious impact on IgA and IgG levels. However, 0.01% *B. licheniformis* significantly decrease IL-1β, IL-6, and TNF-α levels but increase IL-4 secretions. Further, 0.03%

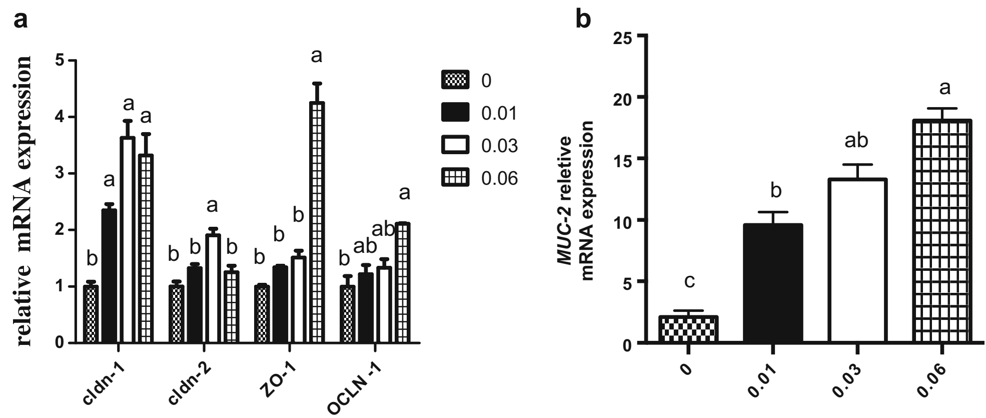

Fig. 1 Effects of *B. licheniformis* supplementation on physical barrier in jejunum of laying hens. Total RNA was extracted and the expressions of **a** *cldn-1*, *cldn-2*, *ZO-1*, and *OCLN-1* and **b** were measured by real-time PCR. Data are expressed as mean ± SEM from three independent experiments. Different letters indicate values significantly different ($P < 0.05$) among the groups

B. licheniformis administration significantly elevates IgG and IL-4 concentrations, but drops the secretions of IL-1β and IL-6. As the dose of *B. licheniformis* increases to 0.06%, IL-1β and IL-6 levels are significantly down-regulated, while IL-4 concentration is enhanced (Table 2). But no significant differences of IgA and IgG are found in the 0.06% *B. licheniformis* group.

Discussion

There is growing evidence that proves that the consumption of probiotics can improve the egg production and egg quality of laying hens [25–27]. In our previous research, *B. licheniformis* administration is able to enhance the laying performance and up-regulate the secretions of hormones and antioxidases [16]. Here, results reinforce the published data on gene level and provide novel evidence for the multifaceted mode of *B. licheniformis* on the egg production and egg quality, including the intestinal barrier and immune functions.

Intestinal epithelia form a functional barrier that separates the intestine from the outside world, and this requires the formation of tight junctions that allow cells to adhere tightly to each other and control the intestinal permeability [28]. Tight junctions are composed of numerous structural and functional proteins, such as CLDN, ZO, and OCLN. It has long been reported that probiotics can play a role in enhancing intestinal barrier functions. VSL#3 probiotics decrease colonic epithelial permeability by increasing expressions of ZO-1 and OCLN in rats [29] and *Escherichia coli* Nissle 1917 also inhibit gut leakage by enhancing ZO-1 expression in mice [30]. Moreover, *Streptococcus thermophilus* and *Lactobacillus acidophilus* can augment the OCLN level to avoid *E. coli* invasion in intestinal epithelial cells [31]. In the present study, we got similar results. The transcript levels of all the tested tight junctions are up-regulated by *B. licheniformis* and this effect is more obvious when *B. licheniformis* is at a higher dosage. Besides, the highly glycosylated mucins secreted by goblet cells also create an important defense line against microbial encroachment [24]. Among them, MUC2, the most abundant mucin, plays an essential part in the organization of the intestinal mucous layers at the epithelial surface of the intestine [32]. Recent report has demonstrated that VSL#3 administration enhances MUC2 secretion and gene expression

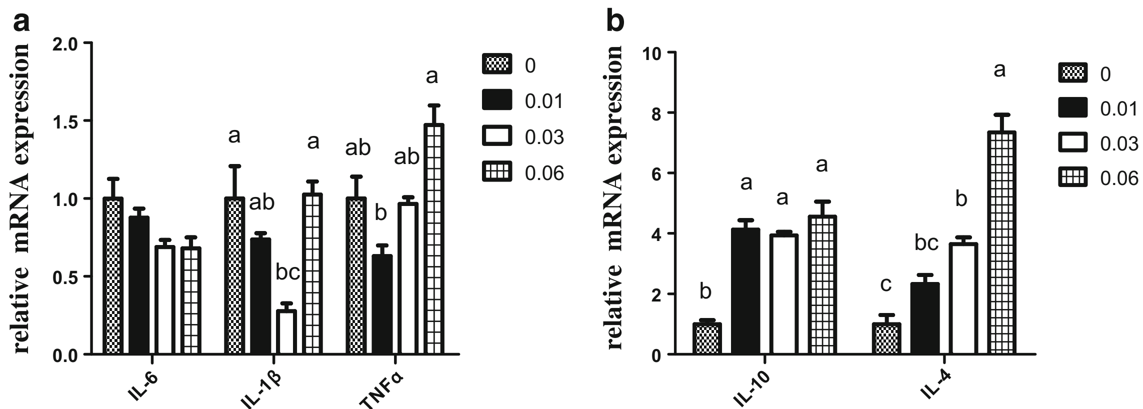

Fig. 2 Effects of *B. licheniformis* supplementation on immunological barrier in jejunum of laying hens. **a** Pro-inflammatory factor mRNA expressions. Total RNA was extracted and the expressions of *IL-6*, *IL-1β*, and *TNF-α* were measured by real-time PCR. **b** Anti-inflammatory factor mRNA expressions. Total RNA was extracted and the expressions of *IL-10* and *IL-4* were measured by real-time PCR. Data are expressed as mean ± SEM from three independent experiments. Different letters indicate values significantly different ($P < 0.05$) among the groups

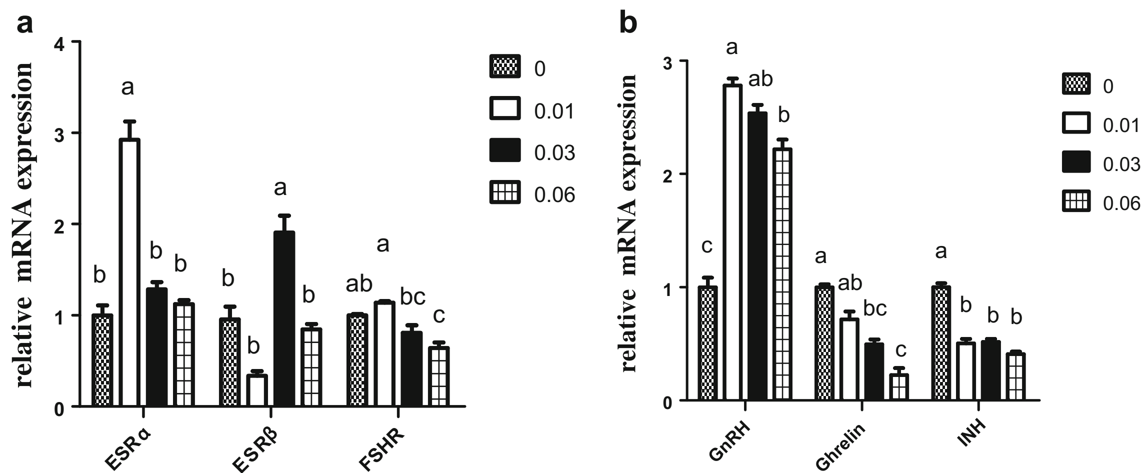

Fig. 3 Effects of *B. licheniformis* supplementation on mRNA expressions of genes encoding hormone receptors (**a**) and hormones (**b**) in ovary of laying hens. Total RNA was extracted and the expressions of *ESRα*, *ESRβ*, *FSHR*, *GnRH*, *Ghrelin*, and *INH* were measured by real-time PCR. Data are expressed as mean ± SEM from three independent experiments. Different letters indicate values significantly different ($P < 0.05$) among the groups

in rat colonic loops effectively [33]. Our results also reveal that *B. licheniformis* treatment dramatically increases jejunal MUC2 gene expression in a dose-dependent manner, implying the inhibition of epithelial cell adherence for enteric pathogens. It is known that compromised intestinal epithelial integrity may facilitate the invasion of endotoxins from gut microbes, resulting in a local imbalance of anti- and pro-inflammatory molecules in the intestines [25]. Thus, the improved gut physical barrier induced by *B. licheniformis* raises the possibility that it may also participate in enhancing the intestinal immunological barrier function. According to Shimazu et al. [34] and Perdigon et al. [35], probiotics down-regulate gene expressions of pro-inflammatory cytokines in porcine intestinal epithelial cells and stimulate the gut immune cells to release IL-4 and IL-10. Our findings are consistent with these findings in that we demonstrate *B. licheniformis* also significantly decrease the expression of pro-inflammatory cytokines IL-1β and TNF-α but increase the transcriptions of anti-inflammatory cytokines IL-4 and IL-10. The main function of anti-inflammatory cytokines is to limit and ultimately terminate inflammatory responses [36]. Over-production of pro-inflammatory cytokines has an adverse effect on intestinal mucosal integrity [37]. Recent studies have indicated that most pro-inflammatory cytokines induce a pathologic opening of the intestinal physical barrier and increase intestinal epithelial permeability [38]. Since it is suggested that intestinal damage may lead to the decreased egg production and quality in chickens [39], we speculate that the enhancement of intestinal, physical, immunological barrier functions caused by *B. licheniformis* can lead to the increase of intestinal health of laying hens, contributing to the improvement of laying performance and egg quality.

Faults in establishing intestinal immunity can lead to disease, inducing local and often also systemic inflammation [40]. Therefore, the enhanced gut immunological barrier inspired us to determine the systemic immunity. Evidence suggest that one of the putative effects of probiotics is the alteration of immune function [41, 42], and in recent decades, probiotics have been used in animals and humans to modulate the humoral immunity in order to enhance the disease resistance capacity [42, 43]. *Lactobacilli* treatment leads to higher antibody production in chickens [44, 45]. Multi-strain probiotics and yeast can increase egg production and egg quality while increasing the antibody titer in serum as well [46]. Our results demonstrate that *B. licheniformis* supplementation significantly increases IgG level in serum, but no

Table 2 Effects of *Bacillus licheniformis* on immunoglobulin and inflammatory factor secretions

B. licheniformis levels (%)	IgA (g/mL)	IgG (μg/mL)	IL-1β (pg/mL)	IL-2 (ng/L)	IL-6 (ng/L)	TNF-α (ng/L)	IL-4 (ng/L)	IL-10 (pg/mL)
0	23.0 ± 4.2	9.5 ± 2.4b	26.6 ± 3.5a	53.1 ± 8	29.6 ± 5.9a	66.9 ± 2.5a	21.1 ± 3.4b	79.6 ± 23.0
0.01	21.1 ± 2.9	11.0 ± 1.6ab	14.5 ± 1.7b	48.7 ± 8.4	16.6 ± 1.1b	49.5 ± 1.3b	60.4 ± 11.7a	61.7 ± 10.8
0.03	24.7 ± 4.6	15.9 ± 3.7a	11.9 ± 0.9b	39.9 ± 8.3	15.0 ± 1.8b	57.7 ± 1.8a	61.4 ± 12.7a	72.1 ± 21.3
0.06	20.2 ± 1.6	12.6 ± 1.6ab	16.1 ± 2.4b	37.6 ± 7.9	17.4 ± 1.6b	61.1 ± 5.4a	66.2 ± 7.8a	99.2 ± 15.1

Data are expressed as mean ± SEM from six independent experiments. Different letters indicate values significantly different ($P < 0.05$) among the groups

significant differences are found for IgA concentration. Further, Klasing reported that excessive amounts of cytokines may decrease feed intake and increase energy expenditure, thereby reducing the performance of livestock [47]. Here, we find that levels of pro-inflammatory cytokines IL-1β and IL-6 are significantly reduced in all the *B. licheniformis* treatment groups. However, TNF-α concentrations are only decreased with 0.01% *B. licheniformis* treatment but returned to normal when the levels of *B. licheniformis* in the diet increase to 0.03 and 0.06%. On the contrary, the levels of anti-inflammatory cytokine IL-4 are markedly induced in all the probiotic groups, although IL-10 is only slightly enhanced when the concentration of *B. licheniformis* reaches to 0.06%. As it has been demonstrated that immunity changes are critical to the laying performance of hens [48, 49], we summarize that *B. licheniformis* can enhance the laying performance of layers by regulating immune function.

In birds, the onset of breeding involves the activation of various hormones. Estrogens play a fundamental role in the regulation of female sexual differentiation and reproduction. ESRs show an appropriate expression profile in the developing embryo [50]. The present study demonstrates that *B. licheniformis* increase ESRα and ESRβ transcript levels, but this effect is not observed in the 0.06% *B. licheniformis* group. FSH is a pituitary glycoprotein hormone, which can stimulate and regulate ovarian follicular development and egg production in chicken. FSH signal transduction is mediated by the FSHR [51]. In this study, 0.01 and 0.03% *B. licheniformis* have no obvious influence on FSHR transcriptions, but 0.06% *B. licheniformis* significantly down-regulate FSHR gene expressions. Although the FSHR expressions are reduced, FSH levels are much higher with *B. licheniformis* treatment [16]. Taken together, *B. licheniformis* can improve egg production and quality via increasing E2, FSH concentrations [16], and *ESR* expressions.

Besides the hormone receptors, we also detected the mRNA expressions of hormone genes *GnRH*, *Ghrelin*, and *INH*. Present findings suggest that *GnRH* gene expressions enhanced with *B. licheniformis* treatments, but *Ghrelin* and *INH* transcriptions decreased. The secretions of GnRH from the hypothalamus stimulate the release of luteinizing hormone (LH) and FSH, which in turn activates gonadal development and release of sex steroids, including E2 and testosterone [52] to regulate reproductive functions [53]. Ghrelin is a gut-brain peptide that functions in the regulation of growth hormone release and food intake [54]. Reports have demonstrated that Ghrelin production can inhibit the secretion of estrogen and GnRH [55, 56]. INH is a dimeric glycoprotein and is important for regulation of dominant follicle development [57]. The deletion of the α-subunit gene of INH markedly elevates serum concentrations of FSH and causes gonadal tumors in immature mice [57–60]. Therefore, the altered expressions of genes encoding GnRH, Ghrelin, and INH caused by *B. licheniformis* are important for the improvement of reproductive performance.

In summary, present experiments indicate that dietary *B. licheniformis* administration improves egg production and egg quality in laying hens via mechanisms as follows: (1) enhancing jejunal physical and immunological barriers, (2) regulating systemic immunity, (3) promoting gene expression of *ESR* and (4) positively modulating expression of genes encoding the hormones that are related to ovulation.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All animal manipulations were conducted in accordance with the guidelines for animal welfare and approved by the Animal Welfare Committee of Animal Science College, Zhejiang University.

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