Effects of fermented feed on growth performance, immune response, and antioxidant capacity in laying hen chicks and the underlying molecular mechanism involving nuclear factor-κB

Fenghua Zhu,[*,a] Beibei Zhang,[*,a] Jin Li,[†] and Lianqin Zhu[†,1]

*College of Animal Science and Technology, Qingdao Agricultural University, Qingdao 266109, People's Republic of China; and †College of Veterinary Medicine, Qingdao Agricultural University, Qingdao 266109, People's Republic of China

ABSTRACT This study investigated the effects of fermented-feed diets on growth performance, immune status, and antioxidant responses in laying hen chicks and the underlying molecular mechanism, specifically, the role of the nuclear factor-κB (**NF-κB**) signaling pathway. A total of 80 healthy 14-day-old laying hen chicks were randomly divided into 4 treatments: basal diet (**CON**); basal diet supplemented with 7.5% fermented feed (**FD**); FD diet plus the NF-κB inhibitor BAY 11-7082 (**FD + BAY**); and FD diet plus the NF-κB inhibitor JSH-23 (**FD + JSH**). The NF-κB inhibitors were administered by intraperitoneal injection. The experiment lasted 21 D. Fermented feed supplementation significantly increased the body weight and average body weight gain of laying hen chicks but significantly decreased the feed conversion ratio. Additionally, fermented feed supplementation significantly increased mitogen-activated T-cell and B-cell proliferation in the peripheral blood, as well as elevated the serum concentrations of interleukin (**IL**)-1, IL-2, IL-4, IL-6, and tumor necrosis factor (**TNF-α**); however, NF-κB inhibition significantly reduced T-cell proliferation and serum IL-1, IL-6, and TNF-α levels. The levels of IgA, IgG, IgM, and Newcastle disease virus antibody in the serum were significantly increased by the addition of fermented feed. Furthermore, fermented feed supplementation significantly improved antioxidant function, as indicated by the increases of total antioxidant capacity, total superoxide dismutase activity, and glutathione peroxidase activity and the decrease of malonaldehyde level. However, NF-κB inhibition reversed these changes. Western blot analysis showed that fermented feed treatment increased splenic IκB kinase β and NF-κB protein levels, whereas these increases were prevented by NF-κB inhibition. In conclusion, fermented feed improves the growth performance, immune function, and antioxidant capacity of laying hen chicks. Fermented feed-induced modulation of T-cell proliferation, T helper type 1 and T helper type 2 cytokine production, and antioxidation is associated with NF-κB activation.

Key words: antioxidation, fermented feed, immune response, laying hen chick, NF-κB

2020 Poultry Science 99:2573–2580
https://doi.org/10.1016/j.psj.2019.12.044

INTRODUCTION

Some meal feeds, such as cottonseed meal and rapeseed meal, contain high levels of crude protein and metabolic energy, which should be valuable sources of plant protein. However, conventional meal feeds contain a variety of antinutritional factors, such as gossypol, phytic acid, tannins, and other toxic factors. In recent years, probiotic fermentation technology has become a powerful tool for reducing antinutritional factors, improving nutritional quality and increasing nutrient bioavailability in animal feed. The fermentation process enhances the concentrations of enzymes and metabolites ， as well as the number of lactobacilli (Stanbury et al., 1995; Song et al., 2010). The lactic acid bacteria in the fermented feed reduce intestinal pH by producing organic acids, and they inhibit the colonization of intestinal pathogens through antagonistic activity, competitive exclusion, and bacteriocin production (Sugiharto and Ranjitkar, 2019). The use of fermented feed improves the weight gain and feed conversion ratio (**FCR**) of broiler chickens (Chiang et al., 2009). Egg weight, shell weight, and shell stiffness in laying hens are increased via fermented feed supplementation (Engberg et al., 2009). Furthermore, providing

Received September 19, 2019.
Accepted December 18, 2019.

[a]The authors contributed equally to the work.

[1]Corresponding author: lqzhu@qau.edu.cn

fermented diet has been demonstrated to increase immune responses, alleviate oxidative stress, improve intestinal morphology, and modulate the gut microbial ecosystem in poultry (Gao et al., 2009; Hu et al., 2016; Yan et al., 2019). Thus, fermented feeds are considered to have beneficial effects on the growth performance and health of animals. However, the mechanisms underlying these effects remain unclear.

The nuclear factor-κB (**NF-κB**) proteins are a family of transcription factors that play crucial roles in inflammation and immune responses (Morgan and Liu, 2011; Oeckinghaus et al., 2011). In addition, NF-κB proteins regulate the expression of numerous genes that are involved in cell proliferation, differentiation, and survival (Morgan and Liu, 2011; Oeckinghaus et al., 2011). The activation of NF-κB is mainly classified into canonical and noncanonical pathways. The canonical pathway is induced by the majority of physiological NF-κB stimuli, such as cytokine receptors, antigen receptors, and pattern-recognition receptors. This pathway is dependent on the catalytically active IκB kinase β (**IKKβ**) and the regulatory subunit IKKγ, leading mainly to the phosphorylation of IκBα and nuclear translocation of mostly p65-containing heterodimers. The noncanonical pathway is stimulated by specific tumor necrosis factor (**TNF**) receptor family members and depends on the IKKα-mediated cleavage of p100 to p52 (Morgan and Liu, 2011). The expression pattern of NF-κB and its effects on various cellular processes have been well documented in studies of lactic acid bacteria. *Lactobacillus crispatus* has been reported to increase the production of TNF-α and interleukin (**IL**)-1β in human monocytic leukemia cell lines, with the increased production being accompanied by NF-κB activation (Klebanoff et al., 1999). The cell wall components of *Lactobacillus* have been shown to bind to immune cell surface pattern-recognition receptors and then potently induce cytokine production directly via the NF-κB signaling pathway (Matsuguchi et al. 2003; Cross et al. 2004). In addition, the metabolites secreted by *Bifidobacterium infantis* and *Lactobacillus acidophilus* both inhibit IκBα degradation and downregulate the nuclear translocation of NF-κB, exerting antiinflammatory effects in mature human fetal enterocytes (Guo et al., 2015).

There is limited information concerning the effects of fermented feed supplementation on the growth performance, immune response, and antioxidant function of laying hen chicks and on the underlying molecular mechanisms. In this study, we assessed the effects of fermented feed supplementation with or without NF-κB inhibition on the growth performance, immune response, and antioxidant activities of laying hen chicks.

MATERIAL AND METHODS

Experimental Design and Bird Management

All of the experimental procedures were approved by the Animal Care and Use Committee of Qingdao Agricultural University. A total of 80 14-day-old White Leghorn specific pathogen-free chickens were randomly assigned to 4 groups (each with 5 replicate cages and 4 birds per cage) for the experiment:

I. CON group, fed a basal diet;
II. FD group, fed a basal diet supplemented with 7.5% fermented feed;
III. FD + BAY group, fed a basal diet supplemented with 7.5% fermented feed and intraperitoneally injected with the NF-κB inhibitor BAY 11-7082 (HY-13453, MedChemExpress, Monmouth Junction, NJ) at 20 mg/kg body weight (**BW**) at 15, 20, 25, 30, and 35 D of age; and
IV. FD + JSH group, fed a basal diet supplemented with 7.5% fermented feed and intraperitoneally injected with the NF-κB inhibitor JSH-23 (HY-13982, MedChemExpress, Monmouth Junction, NJ) at 3 mg/kg BW at 15, 20, 25, 30, and 35 D of age.

The animals in the CON and FD groups were intraperitoneally injected with an equal volume of saline. The doses of BAY 11-7082 and JSH-23 were in accordance with the manufacturer's instructions. There were no significant differences in initial BW among the treatments. All birds were vaccinated using combined Newcastle disease virus and infectious bronchitis virus at 7 D and 21 D of age via intranasal and intraocular administration and using infectious bursal disease virus at 12 D and 27 D of age via the drinking water. Feed and water were provided ad libitum. The ingredient composition and nutrient levels of the diets are shown in Table 1.

Fermented Feed Preparation

Lactobacillus plantarum (CICC20765) and *L. acidophilus* (CICC6006) were obtained from the China Center of Industrial Culture Collection and recovered in sterile Mann-Rogosa-Sharpe (**MRS**) broth medium at 37°C for 16 h with orbital shaking at 160 r/min. After streak cultivation on an MRS agar plate, a single colony was selected for culture in MRS broth medium under the above conditions. For the feed fermentation, 4 parts corn was mixed with 3 parts cottonseed meal and 3 parts rapeseed meal. The *L. plantarum* culture and the *L. acidophilus* culture were both added at a concentration of approximately 5×10^4 colony forming units (**CFU**)/kg of feed. Sterile water was supplemented to achieve 60% moisture content. Then, the wet mixture was transferred to closed plastic bags without air agitation and fermented for 72 h at 32°C. The feedstuffs were fermented and used to formulate the fermented diet every week. After fermentation, the pH value was less than 6.0, and the quantity of lactic acid bacteria was greater than 1×10^9 CFU/kg feed. The basal diet was formulated according to the National Research Council (NRC, 1994).

Growth Performance

BW and feed consumption for each replicate were recorded at 35 D of age. Average daily gain (**ADG**),

Table 1. Ingredients and nutrient composition of the diets.

Items (%, unless otherwise indicated)	Control	Fermented feed
Ingredient		
Corn (8.7% CP)	63.43	59.75
Soybean meal (47.9% CP)	26.50	24.00
Wheat bran	4.00	2.75
Limestone	1.20	1.15
Calcium hydrogen phosphate	1.30	1.30
D,L-Met (98%)	0.15	0.14
L-Lys (98%)	0.12	0.11
Sodium chloride	0.30	0.30
Fishmeal (64.5% CP)	2.00	2.00
Premix[1]	1.00	1.00
Lactic acid bacteria fermented feed	0.00	7.50
Total	100.00	100.00
Nutrient level[2]		
ME (Mcal/kg)	2.85	2.86
CP	19.03	19.09
Calcium	0.92	0.93
Nonphytate phosphorus	0.41	0.40
Lys	0.97	0.98
Met	0.36	0.35

[1]Premix provided the following per kilogram of mixed feed: Mn, 60 mg; Fe, 80 mg; Zn, 60 mg; Cu, 8 mg; I, 0.35 mg; Se, 0.30 mg; vitamin A, 5,000 IU; vitamin D3, 3,000 IU; vitamin E, 20 IU; vitamin B1, 4 mg; vitamin B2, 4 mg; vitamin B5, 20 mg; vitamin B6, 5 mg; vitamin B12, 0.02 mg; niacin, 10 mg; folic acid, 0.8 mg; and biotin, 0.2 mg.
[2]Nutrient levels were calculated by analysis.

average daily feed intake (**ADFI**), and the FCR were calculated during the feeding period.

Sample Collection

At 35 D of age, after 8 h of starvation, 5 birds (1 bird per replicate) were randomly selected from each treatment group. Blood samples were aseptically collected from the wing vein into the EDTA-containing or common vacutainers. The blood samples in the common vacutainers were centrifuged at 3,000 r/min for 15 min at 4°C. Serum samples were obtained and stored at −20°C for cytokine, immunoglobulin (**Ig**), and antioxidant index analyses. The chickens were then killed by jugular exsanguination. The spleen samples were collected, immediately frozen in liquid nitrogen, and stored at −80°C for western blot detection.

Lymphocyte Proliferative Responses

Peripheral blood mononuclear cells were isolated as previously described (Wu et al., 2019) with some modifications. Briefly, EDTA blood samples were diluted 1:1 with sterile PBS and then carefully layered on top of an equal volume of Ficoll lymphocyte separation medium (P8740, Solarbio, Beijing, China). After centrifugation for 20 min at 2,000 r/min, the white flocculent material at the plasma-Ficoll interface was aseptically collected. The lymphocyte suspension was washed 3 times with PBS and then centrifuged at 1,500 r/min for 10 min. Then, the cells were resuspended in RPMI 1640 (Gibco, Carlsbad, CA) complete culture medium supplemented with 5.0% (v/v) fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 10 mM HEPES at final concentration. The number of live cells was counted using trypan blue staining. The cells were adjusted to 5×10^6 cells/mL in RPMI 1640 complete culture medium for the subsequent proliferative responses. The proliferation of T cells and B cells was stimulated with 45 μg/mL concanavalin A (**ConA**; C2613, Sigma Aldrich Co., St. Louis, MO) and 10 μg/mL lipopolysaccharide (**LPS**; L3129, Sigma Aldrich Co.), respectively, and assessed by 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide assay according to the method of Wu et al. (2019). The stimulation index was expressed as a percentage of the absorbance value of stimulated cells relative to the absorbance value of control cells.

Serum Parameter Measurements

The titers of antibodies against Newcastle disease virus (**NDV**) and the levels of total IgG, IgA, and IgM were determined using commercial ELISA kits (RY-12194, RY-12204, RY-12314, and RY-12311; Shanghai Runyu Biotechnology Co., Ltd., Shanghai, China) according to the manufacturer's instructions. The total antioxidant capacity (**T-AOC**), malondialdehyde (**MDA**) level, glutathione peroxidase (**GSH-Px**) activity and total superoxide dismutase (**SOD**) activity were measured using colorimetric kits (RY-12190, RY-12193, RY-12628, and RY-12378; Shanghai Runyu Biotechnology Co., Ltd.) following the manufacturer's guidelines.

Western Blot Analysis

Splenic protein was extracted using RIPA lysis buffer (P0013 B, Beyotime, Shanghai, China) containing protease and phosphatase inhibitor cocktails (P1049, Beyotime). The protein concentration was quantified by the bicinchoninic acid method using a protein assay kit (P0012S, Beyotime). Equal amounts (30 μg) of tissue lysate dissolved in 5 × SDS-PAGE loading buffer (P0015, Beyotime) were heated at 95°C for 10 min and then separated on a 12% SDS-PAGE gel (P0012AC, Beyotime). The separated proteins were electrophoretically transferred to a 0.45 μm polyvinylidene difluoride membrane (FFP24, Beyotime). Nonspecific binding sites were blocked by incubation of the membrane in commercial Quick Blocking Buffer (P0256, Beyotime) at room temperature for 10 min. Then, the membranes were incubated overnight at 4°C with specific primary antibodies: rabbit anti-NF-κB (1: 1,000; ab16502, Abcam, CA, UK), rabbit anti-IKKβ (1:1,000; bs-4880R, Bioss, Beijing, China), and mouse anti-β-actin (1:1,000; Beyotime). After washing 3 times for 5 min with tris-buffered saline containing 0.1% Tween 20 (**TBST**), the membranes were incubated with a horseradish peroxidase-conjugated secondary anti-mouse (A0208, Beyotime) or anti-rabbit (A0216, Beyotime) antibody diluted in TBST (1:3,000) containing 5% nonfat milk for 2 h at room temperature. After washing the membranes 3 times with TBST, the proteins were detected with an ECL Hypersensitive Chemiluminescence kit (P0018FS, Beyotime). Then, signal capture and band density

Table 2. Growth performance of laying hen chicks.

Items[1]	CON	FD	FD + BAY	FD + JSH	SEM	*P*-value
BW (g, day 35)	299.31[b]	326.74[a]	329.53[a]	330.11[a]	3.205	<0.001
ADG (g)	9.39[b]	10.69[a]	10.93[a]	10.91[a]	0.163	0.010
ADFI (g)	30.04	29.93	30.60	30.64	0.255	0.859
FCR (g/g)	3.21[a]	2.81[b]	2.80[b]	2.81[b]	0.037	<0.001

[a,b]Values within the same row with different superscripts were significantly different ($P < 0.05$).

[1]CON, control diet; FD, fermented feed; FD + BAY, fermented feed and intraperitoneally injection with the NF-κB inhibitor, BAY11-7082; FD + JSH, fermented feed and intraperitoneally injection with the NF-κB inhibitor, JSH-23; BW, body weight; ADG; average daily gain; ADFI, average daily feed intake; FCR, feed conversion ratio.

quantification were performed using a Tanon 5200 chemiluminescence image analysis system (Tanon, Shanghai, China). Data were expressed as the ratio of target proteins relative to β-actin.

Statistical Analysis

All data were analyzed by one-way ANOVA and the post hoc Duncan's multiple range test using SPSS statistical software (version 18.0; SPSS Inc., Chicago, IL). $P < 0.05$ was considered statistically significant. The results are expressed as the mean and pooled SEM.

RESULTS

Growth Performance

As shown in Table 2, relative to the control diet, 7.5% fermented feed supplementation significantly increased the BW at 35 D of age ($P < 0.001$) and the ADG from 14 to 35 D of age ($P = 0.01$) but significantly decreased the FCR from 14 to 35 D of age ($P < 0.001$). In the NF-κB inhibition groups, FD + BAY, and FD + JSH, the BW at 35 D of age and ADG, ADFI, and FCR throughout the period were not significantly different from those observed in the FD group ($P > 0.05$).

Lymphocyte Proliferative Response

Relative to the CON group, the 7.5% fermented feed group showed significantly enhanced T-cell and B-cell proliferation, as indicated by the increased ConA and LPS stimulation index in the FD group ($P < 0.001$; Table 3). The T-cell proliferation response in the FD + BAY and FD + JSH groups was significantly inhibited relative to that in the FD group ($P < 0.05$). However, the B-cell proliferative response in the 2 NF-κB blockage groups did not differ from that in the FD group ($P > 0.05$).

Serum Cytokine Concentrations

Serum IL-1, IL-2, IL-4, IL-6, and TNF-α levels in the CON group were significantly lower than those in the FD group ($P \leq 0.05$; Table 4). Birds in the FD + BAY and FD + JSH groups had significantly lower serum levels of IL-1, IL-6, and TNF-α than those in the FD group ($P \leq 0.05$). However, the serum levels of IL-2 and IL-4 in the FD + BAY and FD + JSH groups were comparable to those observed in the FD group ($P > 0.05$).

Serum Total Immunoglobulin Levels and NDV Antibody Titers

As shown in Table 5, the serum levels of IgA, IgG, IgM, and NDV antibody in the FD, FD + BAY, and FD + JSH groups were significantly increased relative to those in the CON group ($P < 0.05$). However, no significant difference was observed among the FD, FD + BAY, and FD + JSH groups ($P > 0.05$).

Serum Antioxidant Parameter Analysis

As shown in Table 6, relative to the control birds, the birds fed fermented feed had significantly elevated serum T-AOC levels and higher total SOD and GSH-Px activities but markedly reduced serum MDA levels ($P < 0.05$). However, these changes were significantly inhibited by the administration of the NF-κB inhibitors, BAY11-7082 and JSH-23, when comparing the FD + BAY and FD + JSH birds with the FD birds ($P < 0.05$).

Splenic Expression of NF-κB and IKKβ Proteins

Relative to the CON group, the FD group had significantly increased splenic protein expression levels of NF-κB and IKKβ ($P < 0.001$; Figure 1). The 2 groups treated with NF-κB inhibitors (FD + BAY and FD + JSH) had significantly lower splenic protein

Table 3. Proliferation of blood lymphocytes of laying hen chicks.

Items[1]	CON	FD	FD + BAY	FD + JSH	SEM	*P*-value
ConA SI	0.45[b]	0.52[a]	0.40[c]	0.40[c]	0.012	<0.001
LPS SI	0.42[b]	0.58[a]	0.61[a]	0.57[a]	0.018	<0.001

[a-c]Values within the same row with different superscripts were significantly different ($P < 0.05$).

[1]CON, control diet; FD, fermented feed; FD + BAY, fermented feed and intraperitoneally injection with the NF-κB inhibitor, BAY11-7082; FD + JSH, fermented feed and intraperitoneally injection with the NF-κB inhibitor, JSH-23; ConA, concanavalin A; LPS, lipopolysaccharides, SI, stimulation index.

Table 4. Serum cytokine levels of laying hen chicks.

Items[1]	CON	FD	FD + BAY	FD + JSH	SEM	*P*-value
IL-1 (ng/L)	15.28[b]	18.46[a]	11.95[c]	11.92[c]	0.620	<0.001
IL-2 (ng/L)	223.93[b]	252.51[a]	248.60[a]	251.77[a]	3.880	0.050
IL-4 (ng/L)	16.97[b]	19.06[a]	18.67[a]	19.03[a]	0.407	0.029
IL-6 (ng/L)	6.54[b]	8.96[a]	4.32[c]	4.30[c]	0.454	<0.001
TNF-α (pg/mL)	11.43[b]	14.81[a]	8.50[c]	7.71[c]	0.666	<0.001

[a-c]Values within the same row with different superscripts were significantly different ($P < 0.05$).

Abbreviation: IL, interleukin.

[1]CON, control diet; FD, fermented feed; FD + BAY, fermented feed and intraperitoneally injection with the NF-κB inhibitor, BAY11-7082; FD + JSH, fermented feed and intraperitoneally injection with the NF-κB inhibitor, JSH-23; TNF-α, tumor necrosis factor-α.

expression levels of NF-κB and IKKβ than the FD group ($P < 0.001$).

DISCUSSION

Fermented feed has attracted increasing amounts of attention because of its potential to improve the nutritional quality of feed and promote animal growth performance (Stanbury et al., 1995; Chiang et al., 2009; Engberg et al., 2009; Yan et al., 2019). Our results showed that relative to control treatment, fermented feed supplementation significantly enhanced BW at 35 D of age and ADG from 14 to 35 D of age but significantly decreased the FCR from 14 to 35 D of age in laying hen chicks. Feed fermentation enriches the contents of probiotics, vitamins, organic acids, amino acids, peptides, enzymes, and growth-promoting factors. This feed processing technology aids the digestion and absorption of nutrients by the host and thereby improve animal growth performance (Stanbury et al., 1995; Peralta et al., 2008). Previous studies have reported that fermentation increased the quality of cottonseed meal and rapeseed meal and thereby promoted growth performance in poultry (Jazi et al., 2017; Drażbo et al., 2019).

Evaluation of the lymphocyte proliferative response to mitogens is an important method for measuring cellular immunity. Our study showed that relative to control treatment, fermented feed supplementation significantly increased ConA-induced T-cell proliferation and LPS-induced B-cell proliferation. Similarly, previous studies reported that the oral administration of some *Lactobacillus* strains induced lymphocyte proliferation in the spleen of cyclophosphamide-immunosuppressed mice (Jang et al., 2013) and the peripheral blood of broilers (Shen et al., 2014). Nuclear factor-κB is a critical nuclear transcription factor in immunity, stress responses, apoptosis, and differentiation. The IKK complex, composed of IKKα and IKKβ, promotes the activation of NF-κB in response to stimuli such as cytokines and antigens (Oeckinghaus et al., 2011). Our results demonstrated that relative to fermented feed supplementation alone, treatment with the NF-κB inhibitors BAY11-7082 and JSH-23 reduced ConA-induced T-cell proliferation in the peripheral blood. Previous studies have demonstrated that mice lacking individual NF-κB proteins have defective B-cell and T-cell proliferation and impaired cytokine production (Sha et al., 1995; Doi et al., 1997; Gerondakis et al., 1998). We speculate that the lack of an effect of NF-κB inhibitor treatment on B-cell proliferation may be because of the effects of downstream or other signaling pathways.

Cytokines play a central role in the cell-mediated immune response. T helper type 1 (**Th1**) cytokines (IL-1, IL-2, TNF-α and interferon) activate cell-mediated immune responses, whereas Th2 cytokines (IL-4, IL-6, and IL-10) promote B-cell growth and differentiation and drive humoral immune responses (Kohut et al., 2001). Our results indicated that the addition of fermented feed to the diet significantly improved the cellular immunity of laying hen chicks, as indicated by the higher serum levels of IL-1, IL-2, and TNF-α and the higher ConA stimulation index in the FD group. The fermented feed supplementation also enhanced the humoral immunity of laying hen chicks, as reflected by the increased serum IL-4 and IL-6 levels and B-lymphocyte proliferation in the FD group. The increased production of cytokines because of fermented feed supplementation may be because of the presence of lactobacilli. The predominant components of

Table 5. Total serum Ig levels and NDV antibody titers of laying hen chicks.

Items[1]	CON	FD	FD + BAY	FD + JSH	SEM	*P*-value
IgA (ng/mL)	106.75[b]	128.00[a]	124.25[a]	121.65[a]	2.216	0.011
IgG (μg/mL)	1.00[b]	1.28[a]	1.30[a]	1.29[a]	0.030	<0.001
IgM (ng/mL)	554.75[b]	649.25[a]	634.50[a]	651.75[a]	9.385	<0.001
NDV-Ab (pg/L)	307.00[b]	358.95[a]	365.29[a]	348.71[a]	5.051	<0.001

[a,b]Values within the same row with different superscripts were significantly different ($P < 0.05$).

[1]CON, control diet; FD, fermented feed; FD + BAY, fermented feed and intraperitoneally injection with the NF-κB inhibitor, BAY11-7082; FD + JSH, fermented feed and intraperitoneally injection with the NF-κB inhibitor, JSH-23; Ig, immunoglobulin; NDV-Ab, Newcastle disease virus antibody titers.

Table 6. Serum antioxidant parameters of laying hen chicks.

Items[1]	CON	FD	FD + BAY	FD + JSH	SEM	*P*-value
T-AOC (U/mL)	0.85[b]	1.10[a]	0.68[c]	0.65[c]	0.046	<0.001
SOD (pg/mL)	4.89[b]	6.15[a]	3.85[c]	3.93[c]	0.244	<0.001
GSH-Px (pmol/mL)	1.30[b]	1.56[a]	1.02[c]	1.16[c]	0.476	<0.001
MDA (nmol/L)	1.74[a]	1.57[b]	1.75[a]	1.78[a]	0.042	0.043

[a-c]Values within the same row with different superscripts were significantly different ($P < 0.05$).

[1]CON, control diet; FD, fermented feed; FD + BAY, fermented feed and intraperitoneally injection with the NF-κB inhibitor, BAY11-7082; FD + JSH, fermented feed and intraperitoneally injection with the NF-κB inhibitor, JSH-23; T-AOC, total antioxidant capability; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; MDA, malonaldehyde.

their cell walls, peptidoglycans, and lipoteichoic acid are immunologically active (Bhakdi et al., 1991; Standiford et al., 1994; Cross et al., 2001). These results are consistent with previous studies showing that *Lactobacillus* strains increased the production of Th1 and Th2 cytokines in cells from both healthy humans and mice (Vissers et al., 2010; Lee et al., 2016).

Compared with fermented feed supplementation alone, BAY11-7082 and JSH-23 administration effectively inhibited the fermented feed-induced production of IL-1, IL-6, and TNF-α. This finding indicates that fermented feed stimulates IL-1, IL-6, and TNF-α production, at least in part, via NF-κB signaling. Nuclear factor-κB has been reported to bind to κB response elements in the promoters and enhancers of target genes, including TNF-α, IL-1, and IL-6 (Miettinen et al., 2000). TNF-α is known to initiate an autoregulatory feedback loop in which the activation of NF-κB induces the production of TNF-α, which further activates NF-κB (Bauerle et al., 1994; Ghosh et al., 1998). To the best of our knowledge, few studies have investigated the role of NF-κB in the regulation of cytokine production induced by fermented feed. However, some studies have reported that the bacterial components of *Lactobacillus* bind to immune cell surface pattern-recognition receptors and then potently induce cytokine production directly via the NF-κB signaling pathway (Matsuguchi et al., 2003; Cross et al., 2004). Previous studies have demonstrated that *L. crispatus* increases the production of TNF-α and IL-1β and activates NF-κB in human monocytic leukemia cell lines (Klebanoff et al., 1999). Jiang et al. (2012) found that *L. acidophilus* upregulates the mRNA levels of cytokines such as IL-1α and IL-1β and that the inhibition of NF-κB significantly reduces these levels in intestinal epithelial cell lines stimulated with *L. acidophilus*.

Serum immunoglobulin concentrations can be used as key indicators of the humoral immunity of animals, as immunoglobulins play key roles in protecting the host against pathogenic viruses and microorganisms (Kong et al., 2007). IgG (IgY), IgM, and IgA are 3 major immunoglobulin classes in avian species (Hanly et al., 1995). In the present study, we observed that dietary fermented feed supplementation improved the humoral immune status of laying hen chicks by increasing their serum levels of IgG, IgA, and IgM. Previous studies demonstrated that the generation of small-sized peptides during fermentation might be associated with elevated serum immunoglobulin concentrations in broiler chickens (Tang et al., 2012; Xu et al., 2012). This conjecture is consistent with the findings of Wang et al. (2003), who reported that dietary supplementation of small peptides at 3 g/kg increased serum immunoglobulin levels in piglets. In the present study, in addition to measuring the systemic humoral

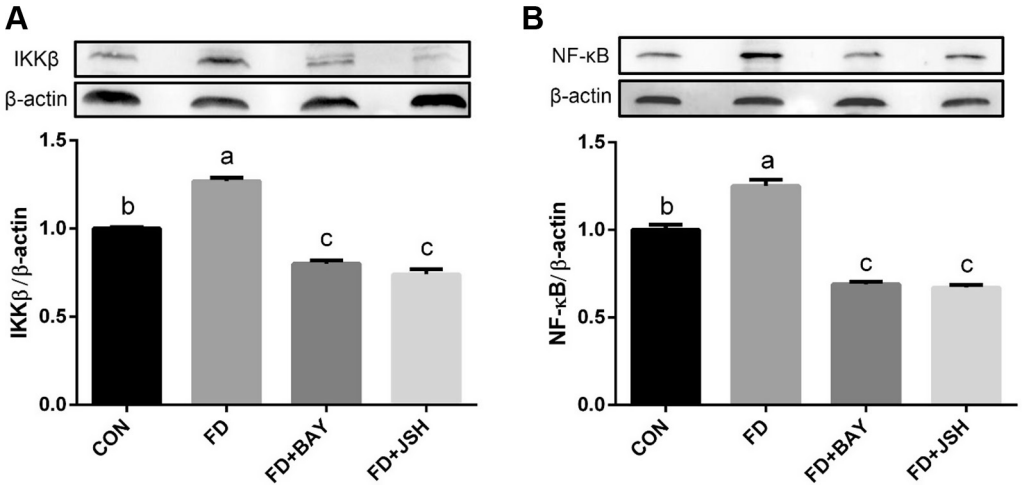

Figure 1. Protein expression of IKKβ (A) and NF-κB (B) in the spleen of laying hen chicks. CON, control diet; FD, fermented feed; FD + BAY, fermented feed and intraperitoneally injection with the NF-κB inhibitor, BAY11-7082; FD + JSH, fermented feed and intraperitoneally injection with the NF-κB inhibitor, JSH-23. IKKβ, IκB kinase β; NF-κB, nuclear factor-κB. Means with different letters were significantly different ($P < 0.05$).

immune status, we determined the immune response capacity to a specific antigen, NDV. Newcastle disease is a highly contagious and widespread global disease in poultry that causes severe economic losses every year (Alexander, 2000). Our data show that fermented feed supplementation significantly increased the serum NDV antibody titer, which indicates that the birds fed fermented feed had a stronger immune response to NDV than did birds of the control group.

Total antioxidant capacity can fully reflect the antioxidant capacity of both enzymatic and nonenzymatic defense systems (Zhang et al., 2018). Antioxidant enzymes, including SOD and GSH-Px, eliminate superoxide anions and hydrogen peroxide in response to oxidative damage (Shen et al., 2014; Zhang et al., 2018). Malondialdehyde produced during lipid peroxidation can be incorporated into proteins via reactions with biomolecules, resulting in the formation of carbonyl derivatives that exert cytotoxic and genotoxic effects (Uchida and Stadtman, 1993). Therefore, the MDA concentration is a marker of lipid peroxidation. Our results showed that fermented feed increased the serum antioxidant capacity of laying hen chicks by increasing SOD and GSH-Px activities and T-AOC levels, as well as decreasing MDA concentration. Similar results have been obtained in other studies using fermented feed (Lim and Lee, 2011; Wu et al., 2015). The inhibition of NF-κB pathway signaling significantly reduces antioxidant index levels after stimulation with fermented feed, suggesting that the activation of NF-κB plays an important role in the increase in antioxidation activity induced by fermented feed. Manganese superoxide dismutase and copper-zinc superoxide dismutase have been shown in numerous studies to be NF-κB targets with antioxidant activity (Jones et al., 1997; Djavaheri-Mergny et al., 2004; Rojo et al., 2004). A sequence analysis revealed that the mouse GSH-Px genes have putative NF-κB binding motifs and that NF-κB actively transactivates GSH-Px in response to oxidative stress (Zhou et al., 2001). The results of our study corroborate these previous findings.

In conclusion, our results indicate that fermented feed improves growth performance, immune function, and antioxidant capacity in laying hen chicks. The roles of fermented feed in modulating T-cell proliferation, Th1 and Th2 cytokine production, and antioxidation are associated with NF-κB activation.

ACKNOWLEDGMENTS

This project was funded by the Shandong Province Agricultural Industry Technology (SDAIT-11-07) and National Key R&D Program of China (2018YFD0501403-2.1).

Conflict of Interest Statement: There are no conflicts of interest to declare.

REFERENCES

Alexander, D. 2000. Newcastle disease and other avian paramyxoviruses. Rev. Sci. Tech. Off. Int. Epizoot. 19:443–455.

Bauerle, P. A., and T. Henkel. 1994. Function and activation of NF-κB in the immune system. Annu. Rev. Immunol. 12:141–179.

Bhakdi, S., T. Klonisch, P. Nuber, and W. Fischer. 1991. Stimulation of monokine production by lipoteichoic acids. Infect. Immun. 59:4614–4620.

Chiang, G., W. Lu, X. Piao, J. Hu, L. Gong, and P. Thacker. 2009. Effects of feeding solid-state fermented rapeseed meal on performance, nutrient digestibility, intestinal ecology and intestinal morphology of broiler chickens. Asian Australas. J. Anim. Sci. 23:263–271.

Cross, M. L., A. Ganner, D. Teilab, and L. M. Fray. 2004. Patterns of cytokine induction by gram-positive and gram-negative probiotic bacteria. FEMS Immunol. Med. Microbiol. 42:173–180.

Cross, M. L., L. M. Stevenson, and H. S. Gill. 2001. Anti-allergy properties of fermented foods: an important immunoregulatory mechanism of lactic acid bacteria? Int. Immunopharmacol. 1:891–901.

Djavaheri-Mergny, M., D. Javelaud, J. Wietzerbin, and F. Besancon. 2004. NF-kappaB activation prevents apoptotic oxidative stress via an increase of both thioredoxin and MnSOD levels in TNFalpha-treated Ewing sarcoma cells. FEBS Lett. 578:111–115.

Doi, T. S., T. Takahashi, O. Taguchi, T. Azuma, and Y. Obata. 1997. NF-kappa B RelA-deficient lymphocytes: normal development of T cells and B cells, impaired production of IgA and IgG1 and reduced proliferative responses. J. Exp. Med. 185:953–961.

Drażbo, A., K. Kozlowski, K. Ognik, A. Zaworska, and J. Jankowski. 2019. The effect of raw and fermented rapeseed cake on growth performance, carcass traits, and breast meat quality in Turkey. Poult. Sci. 98:6161–6169.

Engberg, R. M., M. Hammershoj, N. F. Johansen, M. S. Abousekken, S. Steenfeldt, and B. B. Jensen. 2009. Fermented feed for laying hens: effects on egg production, egg quality, plumage condition and composition and activity of the intestinal microflora. Br. Poult. Sci. 50:228–239.

Gao, J., H. J. Zhang, S. G. Wu, S. H. Yu, I. Yoon, D. Moore, Y. P. Gao, H. J. Yan, and G. H. Qi. 2009. Effect of Saccharomyces cerevisiae fermentation product on immune functions of broilers challenged with Eimeria tenella. Poult. Sci. 88:2141–2151.

Gerondakis, S., R. Grumont, I. Rourke, and M. Grossmann. 1998. The regulation and roles of Rel/NF-κB transcription factors during lymphocyte activation. Curr. Opin. Immunol. 10:353–359.

Ghosh, S., M. May, and Eb. 1998. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Annu. Rev. Immunol. 16:225–260.

Guo, S., Y. Guo, A. Ergun, L. Lu, W. A. Walker, and K. Ganguli. 2015. Secreted metabolites of Bifidobacterium infantis and Lactobacillus acidophilus protect immature human enterocytes from IL-1β-induced inflammation: a transcription profiling analysis. PLoS One 10:e0124549.

Hanly, W. C., J. E. Artwohl, and B. T. Bennett. 1995. Review of Polyclonal antibody production procedures in Mammals and poultry. ILAR J. 37:93–118.

Hu, Y., Y. Wang, A. Li, Z. Wang, X. Zhang, T. Yun, L. Qiu, and Y. Yin. 2016. Effects of fermented rapeseed meal on antioxidant functions, serum biochemical parameters and intestinal morphology in broilers. Food Agric. Immunol. 27:182–193.

Jang, S. E., E. H. Joh, H. Y. Lee, Y. T. Ahn, J. H. Lee, C. S. Huh, M. J. Han, and D. H. Kim. 2013. Lactobacillus plantarum HY7712 ameliorates cyclophosphamide-induced immunosuppression in mice. J. Microbiol. Biotechnol. 23:414–421.

Jazi, V., F. Boldaji, B. Dastar, S. R. Hashemi, and A. Ashayerizadeh. 2017. Effects of fermented cottonseed meal on the growth performance, gastrointestinal microflora population and small intestinal morphology in broiler chickens. Br. Poult. Sci. 58:402–408.

Jiang, Y., X. Lü, C. Man, L. Han, Y. Shan, X. Qu, Y. Liu, S. Yang, Y. Xue, and Y. Zhang. 2012. Lactobacillus acidophilus induces cytokine and chemokine production via NF-κB and p38 mitogen-activated protein kinase signaling pathways in intestinal epithelial cells. Clin. Vaccin. Immunol. 19:603–608.

Jones, P. L., D. Ping, and J. M. Boss. 1997. Tumor necrosis factor alpha and interleukin-1beta regulate the murine manganese superoxide dismutase gene through a complex intronic enhancer involving C/EBP-beta and NF-kappaB. Mol. Cell. Biol. 17:6970–6981.

Klebanoff, S. J., D. H. Watts, C. Mehlin, and C. M. Headley. 1999. Lactobacilli and vaginal host defense: activation of the human

immunodeficiency virus type 1 long terminal repeat, cytokine production, and NF-κB. J. Infect. Dis. 179:653–660.
Kohut, M. L., G. W. Boehm, and J. A. Moynihan. 2001. Moderate exercise is associated with enhanced antigen-specific cytokine, but not IgM antibody production in aged mice. Mech. Ageing Dev. 122:1135–1150.
Kong, X., G. Wu, Y. Liao, Z. Hou, H. Liu, F. Yin, T. Li, R. Huang, Y. Zhang, and D. Deng. 2007. Dietary supplementation with Chinese herbal ultra-fine powder enhances cellular and humoral immunity in early-weaned piglets. Livest Sci. 108:94–98.
Lee, Y. D., Y. F. Hong, B. Jeon, B. J. Jung, D. K. Chung, and H. Kim. 2016. Differential cytokine regulatory effect of three Lactobacillus strains isolated from fermented foods. J. Microbiol. Biotechnol. 26:1517–1526.
Lim, S. J., and K. J. Lee. 2011. A microbial fermentation of soybean and cottonseed meal increases antioxidant activity and gossypol detoxification in diets for Nile tilapia, Oreochromis niloticus. J. World Aquacult. Soc. 42:494–503.
Matsuguchi, T., A. Takagi, T. Matsuzaki, M. Nagaoka, K. Ishikawa, T. Yokokura, and Y. Yoshikai. 2003. Lipoteichoic acids from Lactobacillus strains elicit strong tumor necrosis factor alpha-inducing activities in macrophages through Toll-like receptor 2. Clin. Diagn. Lab. Immunol. 10:259–266.
Miettinen, M., A. Lehtonen, I. Julkunen, and S. Matikainen. 2000. Lactobacilli and streptococci activate NF-κB and STAT signaling pathways in human macrophages. J. Immunol. 164:3733–3740.
Morgan, M. J., and Z.-g. Liu. 2011. Crosstalk of reactive oxygen species and NF-κB signaling. Cell Res. 21:103–115.
National Research Council 1994. Nutrient Requirements of Poultry, 9th rev. edn. Natl. Acad. Press, Washington, DC.
Oeckinghaus, A., M. S. Hayden, and S. Ghosh. 2011. Crosstalk in NF-κB signaling pathways. Nat. Immunol. 12:695–708.
Peralta, E. M., H. Hatate, D. Kawabe, R. Kuwahara, S. Wakamatsu, T. Yuki, and H. Murata. 2008. Improving antioxidant activity and nutritional components of Philippine salt-fermented shrimp paste through prolonged fermentation. Food Chem. 111:72–77.
Rojo, A. I., M. Salinas, D. Martin, R. Perona, and A. Cuadrado. 2004. Regulation of Cu/Zn-superoxide dismutase expression via the phosphatidylinositol 3 kinase/Akt pathway and nuclear factor-kappaB. J. Neurosci. 24:7324–7334.
Sha, W. C., H. C. Liou, E. I. Tuomanen, and D. Baltimore. 1995. Targeted disruption of the p50 subunit of NF-kappa B leads to multifocal defects in immune responses. Cell 80:321–330.
Shen, X., D. Yi, X. Ni, D. Zeng, B. Jing, M. Lei, Z. Bian, Y. Zeng, T. Li, and J. Xin. 2014. Effects of Lactobacillus plantarum on production performance, immune characteristics, antioxidant status, and intestinal microflora of bursin-immunized broilers. Can. J. Microbiol. 60:193–202.
Song, Y., V. Pérez, J. Pettigrew, C. Martinez-Villaluenga, and E. G. de Mejia. 2010. Fermentation of soybean meal and its inclusion in diets for newly weaned pigs reduced diarrhea and measures of immunoreactivity in the plasma. Anim. Feed Sci. Technol. 159:41–49.
Stanbury, P. F., A. Whitaker, and S. J. Hall. 1995. Principles of Fermentation Technology, 2nd ed. Butterworth-Heinemann, Oxford, UK.
Standiford, T. J., D. A. Arenberg, J. M. Danforth, S. L. Kunkel, G. VanOtteren, and R. M. Strieter. 1994. Lipoteichoic acid induces secretion of interleukin-8 from human blood monocytes: a cellular and molecular analysis. Infect. Immun. 62:119–125.
Sugiharto, S., and S. Ranjitkar. 2019. Recent advances in fermented feeds towards improved broiler chicken performance, gastrointestinal tract microecology and immune responses: a review. Anim. Nutr. 5:1–10.
Tang, J. W., H. Sun, X. H. Yao, Y. F. Wu, X. Wang, and J. Feng. 2012. Effects of Replacement of soybean meal by fermented cottonseed meal on growth performance, serum biochemical parameters and immune function of Yellow-feathered broilers. Asian Australas. J. Anim. Sci. 25:393–400.
Uchida, K., and E. Stadtman. 1993. Covalent attachment of 4-hydroxynonenal to glyceraldehyde-3-phosphate dehydrogenase. A possible involvement of intra-and intermolecular cross-linking reaction. J. Biol. Chem. 268:6388–6393.
Vissers, Y. M., J. Snel, P. F. Zuurendonk, B. A. Smit, H. J. Wichers, and H. F. Savelkoul. 2010. Differential effects of Lactobacillus acidophilus and Lactobacillus plantarum strains on cytokine induction in human peripheral blood mononuclear cells. FEMS Immunol. Med. Microbiol. 59:60–70.
Wang, T., Y. Fu, J. Lv, H. Jiang, Y. Li, C. Chen, and C. Yu. 2003. Effects of mini-peptides on the growth performance and the development of small intestines in weaning piglets. Anim. Husbandry Vet. Med. 35:4–7.
Wu, Q. J., Z. B. Wang, G. Y. Wang, Y. X. Li, and Y. X. Qi. 2015. Effects of feed supplemented with fermented pine needles (Pinus ponderosa) on growth performance and antioxidant status in broilers. Poult. Sci. 94:1138–1144.
Wu, Y., W. Zhen, Y. Geng, Z. Wang, and Y. Guo. 2019. Effects of dietary Enterococcus faecium NCIMB 11181 supplementation on growth performance and cellular and humoral immune responses in broiler chickens. Poult. Sci. 98:150–163.
Xu, F. Z., X. G. Zeng, and X. L. Ding. 2012. Effects of replacing soybean meal with fermented rapeseed meal on performance, serum biochemical variables and intestinal morphology of broilers. Asian Australas. J. Anim. Sci. 25:1734–1741.
Yan, J., B. Zhou, Y. Xi, H. Huan, M. Li, J. Yu, H. Zhu, Z. Dai, S. Ying, W. Zhou, and Z. Shi. 2019. Fermented feed regulates growth performance and the cecal microbiota community in geese. Poult. Sci. 0:1–12.
Zhang, S., C. Wang, Y. Sun, G. Wang, H. Chen, D. Li, X. Yu, and G. Chen. 2018. Xylanase and fermented Polysaccharide of Hericium caputmedusae reduce pathogenic infection of broilers by improving antioxidant and anti-inflammatory properties. Oxidative Med. Cell. Longev. 2018:4296985.
Zhou, L. Z., A. P. Johnson, and T. A. Rando. 2001. NF kappa B and AP-1 mediate transcriptional responses to oxidative stress in skeletal muscle cells. Free Radic. Biol. Med. 31:1405–1416.