Research in Veterinary Science 97 (2014) 305–309

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Effects of salinomycin and *Bacillus subtilis* on growth performance and immune responses in broiler chickens

Kyung-Woo Lee [a,b,*], Hyun S. Lillehoj [a], Seung I. Jang [a,1], Sung-Hyen Lee [a,2]

[a] Animal Biosciences and Biotechnology Laboratory, Beltsville Agricultural Research Center, Agricultural Research Service, USDA, Beltsville, MD 20705, USA
[b] Department of Animal Science and Technology, College of Animal Bioscience and Technology, Konkuk University, 120 Neungdong-ro, Gwangjin-gu, Seoul 143-701, South Korea

ARTICLE INFO

Article history:
Received 31 March 2014
Accepted 20 July 2014

Keywords:
Bacillus subtilis
Salinomycin
Growth performance
Immunity
Cytokines
Broiler chickens

ABSTRACT

The present study was undertaken to compare the effect of salinomycin and *Bacillus subtilis* on growth performance, serum antibody levels against *Clostridium* spp. and *Eimeria* spp., and cytokine mRNA expression levels in broiler chickens raised in the used litter. Broiler chickens fed a diet containing salinomycin showed lower ($P < 0.05$) body weights compared with the control diet-fed counterparts. Serum nitric oxide levels were significantly ($P < 0.05$) elevated in chickens fed the *B. subtilis*-enriched diet compared with those on either the salinomycin-fed or control diet-fed chickens. None of the dietary treatments affected ($P > 0.05$) serum antibody levels against *Clostridium perfringens* toxins. Both salinomycin and *B. subtilis* significantly lowered ($P < 0.05$) the serum levels of *Eimeria*-specific antibodies compared with the control group. Salinomycin, but not *B. subtilis*, significantly modulated ($P < 0.05$) the expression of cytokines encoding interferon-γ (IFN-γ), interleukin10 (IL-10) and tumor necrosis factor superfamily 15 (TNFSF15) compared with the control group. In conclusion, dietary salinomycin and *B. subtilis* affected serum anticoccidial antibody and intestinal cytokine expression, but failed to improve growth performance in broiler chickens. Further study is warranted to investigate the mode of action of salinomycin on host immune response and growth performance in broiler chickens.

1. Introduction

Avian coccidiosis is a common parasitic disease of the poultry industry worldwide, and in the United States alone, the cost of anticoccidial medication has been estimated to be $127 million annually (Chapman, 2009). Avian coccidiosis is caused by at least seven distinct species of *Eimeria* apicomplexa protozoa that infect the intestinal mucosa with serious negative consequence on nutrition absorption (Shirley and Lillehoj, 2012). In addition, pre-exposure to certain species of *Eimeria* has been implicated in promoting necrotic enteritis (Williams et al., 2003) and gangrenous dermatitis (Li et al., 2010). Thus, effective control of avian coccidiosis using live coccidia vaccines or coccidiostats is expected to reduce the production losses due to *Clostridium* bacteria (Lee et al., 2011d; Williams, 2005). Due to the recent global trend on drug-free poultry production (Lillehoj and Lee, 2012), development of antibiotic-alternatives for avian coccidiosis control using direct-fed microbials (Lee et al., 2010a, 2010b, 2010c, 2011a) or plant-derived phytochemicals (Lee et al., 2007a, 2007b, 2011c) has been explored. In addition, recent trials have shown that direct-fed microbials in comparison to ionophores (e.g., salinomycin or lasalocid) have an inhibitory effect on *Eimeria* infection (Bozkurt et al., 2014; Giannenas et al., 2012, 2014; Taherpour et al., 2012). However, hardly any studies have been reported to compare the effect of direct-fed microbials or the current commonly used coccidiosis control program on coccidia- or *Clostridium* spp.-related immune parameters in broiler chickens exposed to a field-simulated environment. Thus, we conducted a feeding trial to investigate the effect of two different feed additives, ionophorous antibiotic salinomycin and probiotic *Bacillus subtilis*, on growth performance and serum antibody levels against *Clostridium* spp. and *Eimeria* spp., and cytokine mRNA expression levels in broiler chickens. In order to simulate the commercial setting intended for natural exposure to *Eimeria* or *Clostridium* spp., broiler chickens were raised on the used litter that had been used for at least 10 flocks and that contained *Eimeria* spp. or *Clostridium* spp.

2. Materials and methods

2.1. Experimental design

One hundred and thirty-five 1-day-old male broiler chickens (Ross 708) were purchased from a local hatchery (Mountaire Farms, Millsboro, DE) and received normal vaccinations including Marek's

* Corresponding author. Tel.: +82 2 450 0495; fax: +82 2 452 9946.
E-mail address: kyungwoolee@konkuk.ac.kr (K.-W. Lee).
[1] Current address: Institute of Health and Environment, Daejeon Metropolitan City, Daejeon 305-338, Republic of Korea.
[2] Current address: National Academy of Agricultural Science, Rural Development Administration, Suwon 445-853, Gyeonggi, Republic of Korea.

http://dx.doi.org/10.1016/j.rvsc.2014.07.021
0034-5288/

Table 1
Oligonucleotide primers used for quantitative RT-PCR of chicken cytokines.

Type	RNA target[a]	Primer sequences[b]	PCR product size (bp)	Genbank accession no.
Reference	GAPDH	F: 5′-GGTGGTGCTAAGCGTGTTAT-3′ R: 5′-ACCTCTGTCATCTCTCCACA-3′	264	NM_204305
Proinflammatory	TNFSF15	F: 5′-CCTGAGTATTCCAGCAACGCA-3′ R: 5′-ATCCACCAGCTTGATGTCACTAAC-3′	292	NM_001024578
Th1	IFNγ	F: 5′-AGCTGACGGTGGACCTATTATT-3′ R: 5′-GGCTTTGCGCTGGATTC-3′	259	NM_205149
Th2	IL10	F: 5′-CGGGAGCTGAGGGTGAA-3′ R: 5′-GTGAAGAAGCGGTGACAGC-3′	272	NM_001004414

[a] GAPDH = glyceraldehyde 3-phosphate dehydrogenase; IL = interleukin; TNFSF = tumor necrosis factor superfamily; IFN = interferon; Th1 = T helper type 1; Th2 = T helper type 2.
[b] F, forward primer; R, reverse primer.

disease (turkey herpes virus (HVT), SB-1 and Rispen) *in ovo* at 18 days of incubation, and Newcastle disease and infections bronchitis via coarse spray after hatch at the hatchery. Upon arrival, they were randomly allotted into one of nine pens (1 m × 1 m) with used litter as bedding and stocking density was set at 0.06 m^2 per bird. No abnormal physical property of used litter employed in this study was noticed. The temperature of facility was maintained at 32 °C during the first week posthatch and gradually decreased to reach 25 °C at 3 weeks. Continuous light was used throughout the experimental period.

The present experiment consisted of three dietary treatments which were given for 28 days. Each treatment was consisted of three pens and each pen had 15 chickens ($n = 45$ chickens/treatment). There were three experimental diets; control diet; diet + 1.5×10^5 cfu/g of *B. subtilis* (Avicorr™, Danisco/Agtech Products Inc., Waukesha, WI); 60 mg/kg of salinomycin (Bio-Cox, Alpharma Inc., Fort Lee, NJ). Basal diet was in a nonmedicated mash form consisting of corn, soybean meal, poultry and animal by-product, and distiller's dried grains soluble and used as a control diet. Experimental diet was formulated by mixing the basal diet with 500 mg of *B. subtilis* (3×10^{11} cfu/g of product) or salinomycin premix (0.12% salinomycin sodium as active component in the premix) to reach 1.5×10^5 cfu/g of *B. subtilis* or 60 mg/kg of salinomycin. In order to ensure the homogeneity of the additives, approximately 3 kg of the basal diet added with the additive were thoroughly mixed using air-tight bucket. Diet and water were provided *ad libitum*, and all experimental protocols were approved by the Small Animal Care Committee of Beltsville Agricultural Research Center.

2.2. Sampling

At day 28, body weight was individually recorded and five birds per pen from each treatment group were randomly sampled for blood after euthanasia. Sera were obtained by gentle centrifugation and stored at –20 °C until use. Immediately after blood sampling, 5-cm segments from mid-jejunum and mid-ileum were sampled, pooled by pen and used for RNA isolation for measurement of cytokine mRNA levels.

2.3. Nitric oxide determination

Serum (100 µl) was mixed with an equal volume of freshly prepared Griess reagent (Sigma, St. Louis, MO) containing 1% (wt/vol) sulfanilamine in 5% phosphoric acid and 0.1% N-naphthylethylenediamine, the mixture was incubated for 10 min at room temperature, and the optical density at 540 nm was determined with an automated microtiter plate reader. Nitrite concentrations were calculated from a standard curve generated with $NaNO_2$.

2.4. Antibody levels against Eimeria spp. or Clostridium perfringens

Serum antibodies against *Eimeria* spp. and *C. perfringens* were measured by in-house ELISAs using recombinant *Eimeria* profilin or EtMIC2 (Lillehoj et al., 2005a, 2005b; Lee et al., 2011a) and *C. perfringens* alpha toxin or necrotic enteritis B-like (NetB) toxin (Lee et al., 2011b, 2012b). The profilin gene was originally isolated from *Eimeria acervulina* and known to be expressed by sporozoites and merozoites of *Eimeria tenella*, *E. acervulina*, and *Eimeria maxima*. EtMIC2 was originally cloned from *E. tenella* and shown to encode a microneme adhesion involved in parasitic motility and host cell invasion by the parasite. *C. perfringens* α-toxin and NetB toxin were expressed in *Escherichia coli* and their corresponding recombinant proteins were purified as described (Lee et al., 2011b, 2012b). For ELISA assay, separate 96-well microtiter plates were coated overnight with 1 µg/well of purified recombinant proteins. The plates were washed with PBS containing 0.05% Tween (PBS-T), and blocked with PBS containing 1% BSA for 1 h at room temperature. Then, diluted serum samples were added (100 µl/well), incubated with gentle agitation for 2 h at room temperature, and washed with PBS-T. Bound antibodies were then detected with peroxidase-conjugated rabbit anti-chicken IgY (Sigma) and peroxidase specific substrates. A value of OD_{450} was determined with a microplate reader (Bio-Rad, Richmond, CA).

2.5. Cytokine mRNA assay

Total RNA was extracted from pooled intestines of five birds per pen ($n = 3$ replicates per treatment) using TRIzol (Invitrogen) as described (Lee et al., 2013). In brief, 5 µg of total RNA were treated with 1.0 U of DNase I and 1.0 µl of 10X reaction buffer (Sigma), incubated for 15 min at room temperature, 1.0 µl of stop solution was added, and the mixture was heated at 70 °C for 10 min. RNA was reverse transcribed using the StrataScript first-strand synthesis system (Stratagene, La Jolla, CA) according to the manufacturer's recommendations. Quantitative RT-PCR oligonucleotide primers for chicken cytokines and GAPDH are listed in Table 1. Amplification and detection were carried out using equivalent amounts of total RNA from intestine using the Mx3000P system and Brilliant SYBR Green QPCR master mix (Stratagene) as described (Lee et al., 2013). Standard curves were generated using $\log_{10}$ diluted standard RNA and the levels of individual transcripts were normalized to those of GAPDH using the Q-gene program (Muller et al., 2002). Each analysis was performed in triplicate. To normalize RNA levels between samples within an experiment, the mean threshold cycle (C_t) values for the amplification products was calculated by pooling values from all samples in that experiment.