

Transcriptional profiling of cecal gene expression in probiotic- and *Salmonella*-challenged neonatal chicks

S. E. Higgins,*¹ A. D. Wolfenden,† G. Tellez,† B. M. Hargis,† and T. E. Porter*¹

*Department of Animal and Avian Sciences, University of Maryland, College Park 20742; and †Center of Excellence for Poultry Science, University of Arkansas, Fayetteville 72701

ABSTRACT Probiotics are currently used to improve health and reduce enteric pathogens in poultry. However, the mechanisms by which they reduce or prevent disease are not known. *Salmonella* are intracellular pathogens that cause acute gastroenteritis in humans, and infections by nontyphoid species of *Salmonella* also can result in diarrhea, dehydration, and depression in poultry. Frequently, however, no clinical signs of infection are apparent in poultry flocks. In this study, day-of-hatch chicks were challenged with *Salmonella enterica* serovar Enteritidis (SE) and treated 1 h later with a poultry-derived, *Lactobacillus*-based probiotic culture (FloraMax-B11, Pacific Vet Group USA Inc., Fayetteville, AR). Cecae were collected 12 and 24 h posttreatment for *Salmonella* detection and RNA isolation for microarray analysis of gene expression. At both 12 and 24 h, SE was significantly reduced in chicks treated with the probiotic as compared with the birds challenged

with only SE ($P < 0.05$). Microarray analysis revealed gene expression differences among all treatment groups. At 12 h, 170 genes were expressed at significantly different levels ($P < 0.05$), with a minimum difference in expression of 1.2-fold. At 24 h, the number of differentially regulated genes with a minimum 1.2-fold change was 201. Pathway analysis revealed that at both time points, genes associated with the nuclear factor kappa B complex, as well as genes involved in apoptosis, were significantly regulated. Based on this analysis, probiotic-induced differential regulation of the genes growth arrest-specific 2 (*GAS2*) and cysteine-rich, angiogenic inducer, 61 (*CYR61*) may result in increased apoptosis in the cecae of chicks. Because *Salmonella* is an intracellular pathogen, we suggest that increased apoptosis may be a mechanism by which the probiotic culture reduces *Salmonella* infection.

Key words: chicken, *Salmonella*, probiotic, gene expression, microarray

2011 Poultry Science 90:901–913
doi:10.3382/ps.2010-00907

INTRODUCTION

In the United States, it is estimated that 1.4 million humans contract salmonellosis and that the cost of this illness, including lost productivity, is \$3 billion annually (World Health Organization, 2006). Another estimate places the cost of salmonellosis from only poultry-related serovars at \$966 million in the United States alone (Callaway et al., 2008). In the year 2006, surveillance data indicated that *Salmonella* caused the greatest number of food-borne illnesses, comprising 38.6% of all laboratory diagnoses (Centers for Disease Control and Prevention, 2009). These surveillance data also indicate that *Salmonella* infections in humans have not decreased significantly since 1996 to 1998, when food-borne illness data began to be collected in the United

States. Because poultry and poultry products often serve as the vehicle for human salmonellosis (Kimura et al., 2004; Marcus et al., 2007), the poultry industry and governmental agencies are focused on reducing *Salmonella* both in live birds and at the processing plant (Hargis et al., 2001; Callaway et al., 2008).

Because chicks are most susceptible to *Salmonella* infection at hatch, and specific isolates of *Salmonella* found in the hatchery have likewise been isolated at processing (Bailey et al., 2002; McCrea et al., 2006), it is widely accepted that reduction of *Salmonella* pre-slaughter will result in reductions during processing and in the resulting poultry products. Corrier et al. (1998) showed that administration of a competitive exclusion culture at hatch significantly reduced *Salmonella enterica* serovar Typhimurium (ST) in chickens just before processing, and Bailey et al. (2000) demonstrated in a field trial that competitive exclusion treatment at hatch resulted in fewer *Salmonella*-positive carcasses in treated flocks at processing. Yet our understanding of how probiotics mediate these health benefits, specifi-

©2011 Poultry Science Association Inc.

Received May 20, 2010.

Accepted October 25, 2010.

¹Corresponding authors: stacyerinhiggins@gmail.com and tporter@umd.edu

Table 1. Description of treatment groups administered to neonatal chicks

Treatment group	Challenge ¹	Treatment ²
Control	Sterile saline	Skim milk
B11	Sterile saline	1.1×10^7 cfu of B11
SE	4.5×10^3 cfu of SE	Skim milk
SE + B11	4.5×10^3 cfu of SE	1.1×10^7 cfu of B11

¹*Salmonella enterica* serovar Enteritidis bacteriophage type 13A (SE; USDA National Veterinary Services Laboratory, Ames, IA) diluted in sterile saline was administered by oral gavage at the indicated concentration in a 0.25-mL volume using an animal feeding needle.

²FloraMax-B11 (B11; Pacific Vet Group USA Inc., Fayetteville, AR) was diluted in nonfat milk and administered by oral gavage 1 h postchallenge at the indicated concentration in a 0.25-mL volume using an animal feeding needle.

cally the reduction of *Salmonella* infection, is very limited.

Previously, it was thought that administration of bacteria such as probiotics to neonates directly reduced infection by pathogens because of “competitive exclusion” between the bacteria. Competitive exclusion was first described by Rantala and Nurmi (1973), who suggested that bacteria compete with each other for space and nutrients. Their data indicated that early administration of “good” bacteria prevented infection by pathogens. Currently, it is also understood that the commensal bacteria can prevent infection by pathogens through maintenance of the integrity of the gastrointestinal tract and regulation of the immune system, and that administration of probiotic bacteria may also facilitate these benefits (Patterson and Burkholder, 2003; Higgins et al., 2005; Revollo et al., 2006; Brisbin et al., 2008b). Using a well-established SE challenge model, we previously observed an increase in SE incidence in cecal tonsils over the initial 12 h posttreatment (Higgins et al., 2007), which indicates that the SE were continuing to cause infection despite theoretically coming into contact with the probiotic organisms within approximately 2 h (neonatal chick gastrointestinal transit time). In the present study, we hypothesized that the probiotic treatment would influence the host chick to reduce or prevent infection. Therefore, the present experiment was designed to characterize global changes in gene expression at early time points within the cecae, a primary site of gastrointestinal colonization in chickens, following challenge with SE and treatment with a poultry-derived *Lactobacillus*-based probiotic culture (B11; FloraMax-B11, Pacific Vet Group USA Inc., Fayetteville, AR).

MATERIALS AND METHODS

Birds

Day-of-hatch chicks were obtained from a local broiler breeder hatchery and transported to the laboratory. Chicks were randomized and assigned to 1 of 4 treatment groups (n = 40; Table 1). The chicks were housed

in battery brooders and maintained at age-appropriate temperatures. They were provided a standard chick starter ration, containing no antimicrobial additives, and water ad libitum. These experiments were reviewed and approved by both the University of Maryland and University of Arkansas institutional animal care and use committees.

Salmonella enterica Serovar Enteritidis Bacteriophage Type 13A

The challenge organism for this experiment was a primary poultry isolate of *Salmonella enterica* serovar Enteritidis, bacteriophage type 13A (SE), which was obtained from the USDA National Veterinary Services Laboratory (Ames, IA). This isolate is resistant to novobiocin (NO; 25 µg/mL) and was selected for resistance to naladixic acid (NA; 20 µg/mL) within the Hargis laboratory. The SE was grown by passaging in tryptic soy broth every 8 h for 24 h, washing by centrifugation in sterile saline, and estimating concentration by using a spectrophotometer. Challenge with SE was administered to 2 groups of chicks on arrival at the laboratory by oral gavage (4.5×10^3 cfu in 0.25 mL) using an animal feeding needle.

Probiotic Culture

The B11 (Pacific Vet Group USA Inc.) was a lactic acid bacteria-based probiotic culture (including *Lactobacillus* spp. and *Pediococcus* spp.) derived from poultry. At 1 h postchallenge, the probiotic culture was diluted in skim milk and administered (1.1×10^7 cfu in 0.25 mL) to 1 challenged and 1 unchallenged group of chicks by oral gavage. Administration of the live B11 probiotic culture at this concentration has consistently resulted in significant reductions in SE infection by 24 h in previous experiments (Higgins et al., 2008, 2010). The other 2 treatment groups simultaneously received the skim milk alone (Table 1).

Previous work has established that selected probiotic cultures, and specifically B11, are effective for the reduction of SE in poultry (Higgins et al., 2005, 2007, 2008; Vicente et al., 2007, 2008). The present model using an SE challenge on the day of hatch was developed previously to simulate a natural infection of neonatal chicks obtained from the hatchery, which is generally accepted as the most frequent source of *Salmonella* contamination in poultry. The selected dose of this isolate of *Salmonella* is effective for obtaining both intestinal and systemic infection in approximately 80% of challenged chicks, and is reasonably consistent with a challenge dose that the chicks might see from consumption of fecal material from infected chicks. The probiotic treatment administered 1 h postchallenge was designed to simulate the treatment of chicks at the time of arrival and placement on a farm. Recently, Higgins et al. (2007) designed time-course experiments to determine

SE infection using this probiotic, and it was revealed that reduction of SE occurs consistently between 12 and 24 h.

Sample Collection

Twelve hours and 24 h posttreatment, 20 chicks per treatment were killed, and both ceca were aseptically removed from each chick. Two ceca (1 ceca each from 2 chicks) were pooled in a sterile sample bag for recovery of SE. The other cecum from each chick was incised longitudinally and rinsed thoroughly with sterile saline. Two rinsed ceca samples were pooled (8 pools collected per treatment group) and immediately snap frozen in liquid nitrogen. Frozen samples were stored at -80°C until RNA extraction.

Salmonella Recovery

Cecal samples from SE-challenged groups were homogenized manually, diluted 1:10 in sterile saline, and then serially diluted. Appropriate dilutions were spread-plated onto xylose lysine deoxycholate agar plates containing NO (25 $\mu\text{g}/\text{mL}$) and NA (20 $\mu\text{g}/\text{mL}$) for enumeration of SE. Plates were incubated for 18 to 24 h at 37°C , and colonies were counted. The first diluted cecal sample was also enriched by the addition of an equal volume of $2\times$ tetrathionate broth, and incubated overnight at 37°C . Twenty cecal samples from nonchallenged chicks (groups 1 and 2) were also enriched with tetrathionate broth to confirm that no SE was present in the unchallenged groups. Following incubation, these samples were streaked for isolation onto xylose lysine deoxycholate agar plates containing NO (25 $\mu\text{g}/\text{mL}$) and NA (20 $\mu\text{g}/\text{mL}$) and incubated for 18 to 24 h at 37°C . Plates were evaluated for the presence or absence of SE, which grow as black colonies on this selective medium.

Microarray Analysis

Microarrays consisting of 21,120 oligonucleotide features were obtained for these studies from the Genomics Research Laboratory at the Steele Children's Research Center at the University of Arizona in Tucson (<http://www.grl.steelecenter.arizona.edu/products.asp>). This array was developed by ARK-Genomics (<http://www.ark-genomics.org/microarrays/bySpecies/chicken/>) using chicken ENSEMBL transcripts and covers much of the chicken genome. Annotation of this array is available at Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc=GPL6049>). Four slides, each consisting of an individual pooled sample (4 biological replicates), were hybridized per treatment group and time point; 32 slides were hybridized in all. Total cellular RNA was isolated from the pooled cecae using an RNeasy Midi Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Quantification was accomplished using the Ribogreen

assay (Invitrogen, Carlsbad, CA), and quality was evaluated by visualizing samples in a formaldehyde gel.

Two micrograms of pooled total RNA from 2 cecal samples were then used for amplification of the mRNA using a modification of the Eberwine procedure (Phillips and Eberwine, 1996) using an Amino Allyl MessageAmp II aRNA Amplification Kit (Ambion, Austin, TX). Briefly, reverse transcription was performed using an oligo dT primer containing a T7 promoter. The purified cDNA provided a template for in vitro transcription, which resulted in antisense amplified copies of mRNA containing the modified nucleotide 5-(3-aminoallyl)-uridine triphosphate. Samples consisting of 20 μg of antisense RNA (**aRNA**) were labeled with Alexa Fluor dyes (Invitrogen) and purified. Following analysis with a NanoDrop spectrophotometer to determine concentration, 8 μg of purified labeled aRNA was hybridized to the microarrays using a reference design (Simon et al., 2002).

An internal reference standard, created by pooling aRNA from all samples within the experiment, was labeled with Alexa Fluor 647. Experimental samples labeled with Alexa Fluor 555 were hybridized to individual microarrays along with the Alexa Fluor 647-labeled reference pool. Labeled aRNA samples, plus 25 μg of yeast transfer RNA and 25 μg of salmon testes DNA (Sigma, St. Louis, MO), were hybridized to microarray slides overnight at 42°C in microarray hybridization buffer (Roche Diagnostics Corporation, Indianapolis, IN). Following hybridization, slides were washed carefully with increasing stringency using sodium citrate and scanned with a 418 confocal laser scanner (Affymetrix, Santa Clara, CA) at 555 nm for Alexa Fluor 555 and at 647 nm for Alexa Fluor 647. Two TIFF images were obtained for each slide.

The data were analyzed according to established protocols in the Porter laboratory as described previously (Ellestad et al., 2006). Images were initially analyzed using GenePix Pro 6.0 software (Molecular Devices, Sunnyvale, CA). The numeric data were then exported for data normalization using a Microarray Data Analysis System (MIDAS, version 2.18; Saeed et al., 2003). Data from the Alexa Fluor 555 channel (the experimental sample) were Lowess normalized by block without background correction, followed by SD regularization first by block and then by slide, by using the Alexa Fluor 647-labeled pool as a reference. The \log_2 ratio (normalized Alexa Fluor 555/Alexa Fluor 647; sample/reference pool) for each spot was calculated.

Before statistical analysis, Lowess-normalized data were trimmed as follows. First, all results for individual genes on each array returning greater than 90% saturated pixel intensities and pixel intensities less than 3 times the background were eliminated. Second, all genes missing more than 8 data points (25% of total) were discounted from further analysis. The resulting trimmed data were analyzed statistically using a one-way ANOVA (SAS Institute, Cary, NC) to compare gene expression between treatment groups. Spots

Table 2. Primer sequences used for quantitative real-time reverse-transcription PCR

RIGG no.	Gene name ¹	ENSEMBL ID (ENSGALG0000_)	Forward sequence	Reverse sequence
12610	<i>PLAU</i>	05086	AACATGGTCTGTGCTGGAGA	AATCCCATAAAGCGTCATCC
16089	<i>IFIH1</i>	11089	ACAGGACGTTGCAAGACAAG	GGTCAAGACCTCGATAAAACCA
11896	<i>ATP2A2</i>	03835	GGGAGAATATCTGGCTGGTG	TAAAGGCGTGATCTGGAAGA
06327	<i>GAS2</i>	03655	CTCCTTGCAAATGCCCTAAT	ATGCTTGTGTGAAGCATCC
00598	<i>RNF130</i>	05820	AGCCACAGCTAGCTTGAATG	GTGCTGCCAGTTCGATAAAT
09550	<i>GCLC</i>	16313	AGGTGGATGTGGACACAAGA	CGATGAATTCCCTCATCCAT
17383	<i>HTRA1</i>	09546	GTTGTCTCTGGGGCCTATGT	TCGACTGTCCATTGATGCTT
00005	<i>HES1</i>	02055	GGACATCTGGAGATGACG	TCATGCACTCATTGAAACCA
14634	<i>CYR61</i>	08661	CCCAGCTACGCCCTCCCTGAAG	GGGGCGGTACTTCTTCACACTGG
07243	<i>FAS</i>	06351	ACACAGCTGCAGCAGACACT	CTCACAATGTCAGGGACGTG
17920	<i>SCL34A2</i>	14372	GAGCGTTCTTATCCCCTCAC	AAAAAGTGGCACAAAGGCAAT
19267	<i>GLL2</i>	16669	CTCTCCTCTTCTGGCACTC	TTGATTAGATGGCTGGGACA
11677	<i>AKR1B1</i>	19283	CAGCTTCAACAGGAATTGGA	TGAAGCCAGGTTACAAACA
06784	<i>14774</i>	23757	CAGCCAGTATGTCTTTGTGGA	GTTTCCCAAGGTCTCCTTCA
10457	<i>CYP1A4</i>	01325	CATGCTGCCCTACACAGAAG	TCCTTGGGGATGTAATAGCC

¹*PLAU* = urokinase-type plasminogen activator; *IFIH1* = interferon induced with helicase-1; *ATP2A2* = sarcoplasmic/endoplasmic reticulum calcium adenosine triphosphatase 2; *GAS2* = growth arrest-specific 2; *RNF130* = ring finger protein 130; *GCLC* = glutamate-cysteine ligase catalytic subunit; *HTRA1* = serotonin 5-HT1a; *HES1* = hairy and enhancer of split-1; *CYR61* = cysteine-rich, angiogenic inducer, 61; *FAS* = tumor necrosis factor receptor superfamily, member 6; *SCL34A2* = type IIb sodium phosphate cotransporter; *GLL2* = gallinacin 2; *AKR1B1* = aldo-keto reductase family 1, member B1; *14774* = uncharacterized (ortholog of transmembrane protein 199); *CYP1A4* = cytochrome P450, family 1, subfamily A, polypeptide 4.

determined to be statistically significant ($P < 0.05$) among treatment groups were analyzed further. The data from this experiment have been deposited in the Gene Expression Omnibus as Accession no. GSE19887 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE19887>).

Pathway Analysis

The genes considered to be differentially regulated because of treatment within a time point (missing no more than 25% data points, $P < 0.05$, and a fold difference minimum of 1.2) were further analyzed using Ingenuity Pathway Analysis software (<http://www.ingenuity.com>). Putative gene networks were generated to predict interactions between differentially regulated genes.

Quantitative Real-Time Reverse-Transcription PCR

Quantitative real-time reverse-transcription PCR (qRT-PCR) was performed on 12 genes to confirm gene expression patterns observed in the microarray analysis. All 8 individual samples were used for the qRT-PCR analysis. Two-step qRT-PCR was performed on all 64 samples. Each reaction included 0.5 μ g of total RNA, an oligo dT primer (5'-CGGAAT-TCTTTTTTTTTTTTTTTTTTTTTT-3'; Sigma Genosys, St. Louis, MO), and Superscript III reverse transcriptase and RNase Out RNase inhibitor (Invitrogen). A negative control for genomic DNA contamination was prepared by pooling RNA from each sample and using 0.5 μ g in a reaction without addition of Superscript III. All first-strand cDNA reactions were diluted 5-fold before use in PCR reactions.

Polymerase chain reaction primers were designed with the Primer 3 program (Rozen and Skaletsky, 2000), available for use online (<http://fokker.wi.mit.edu/primer3/input.htm>), using the full-length mRNA sequence predicted from the chicken genome available through ENSEMBL (http://www.ensembl.org/Gallus_gallus/index.html) as template. The primer sequences used in these experiments are detailed in Table 2. Messenger RNA levels were quantified using a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad) and a 2 \times Quantitect SYBR Green PCR Master Mix (Bio-Rad). Polymerase chain reaction was performed as follows: denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Dissociation curve analysis and gel electrophoresis were used to ensure that a single PCR product was amplified in each reaction. Data were normalized to the housekeeping gene β -actin (*ACTB*), and data were transformed using the equation 2^{-Ct} , where Ct represents the fractional cycle number when the amount of amplified product reached a threshold for fluorescence. Data were divided by the mean of the expression levels of the control treatment at each time point for statistical analysis and comparison with microarray results. Results were then analyzed statistically (ANOVA) to confirm statistically significant effects of treatments, as described previously (Ellestad et al., 2006).

RESULTS

We recovered significantly less SE from chicks treated with SE + B11 at both 12 and 24 h posttreatment, relative to SE treatment alone (Table 3). At 12 h, there was a 40% reduction in SE-infected chicks and a 4-log₁₀ reduction in numbers of detectable SE. At 24 h, we observed a 50% reduction in numbers of SE-infected chicks and a 3-log₁₀ reduction in the numbers of detect-

able SE in the cecae. No SE was detected after enrichment in the control or B11 treatment groups, indicating no incidental contamination of SE between treatment groups.

Microarrays were performed using RNA obtained from the rinsed cecae from 8 chicks (4 pools of 2) at each time point. When data were compared across all 4 groups at a single time point, 309 genes were expressed at significantly different levels at 12 h, and 352 genes were significantly different in expression at 24 h. Among those, 170 genes had a minimum of a 1.2-fold difference among treatment groups at 12 h (Supplemental Table 1; <http://ps.fass.org/content/vol90/issue4>), and 201 genes had a minimum of a 1.2-fold difference among treatment groups at 24 h (Supplemental Table 2; <http://ps.fass.org/content/vol90/issue4>). When the SE and SE + B11 treatment groups were compared, 6 genes were upregulated in the SE + B11 group at 12 h, and 11 genes were downregulated in the SE + B11 treatment group. At 24 h, 32 genes were upregulated by SE + B11 and 25 genes were downregulated by SE + B11 compared with SE.

Quantitative real-time PCR was performed on genes selected based on the gene function and the fold difference between treatment groups (Figure 1). Genes regulated by SE included solute carrier family 34 member 2 (*SLC34A2*) and gallinacin 2 (*GLL2*), which were both increased in the cecae of SE-challenged chicks at 12 h, but not at 24 h, as compared with the control. Aldoketo reductase family 1, member B1 (*AKR1B1*) was increased by SE at 24 h only. Interestingly, expression of cytochrome P450, family 1, subfamily A, polypeptide 4 (*CYP1A4*) was reduced by SE in both challenged groups at both time points. One gene, *14774*, is currently uncharacterized but is an ortholog of transmembrane protein 199 in other species. Expression of this gene was increased by B11 only at 24 h.

Genes that were significant and with at least a 1.2-fold difference in expression levels among groups were analyzed using Ingenuity Pathway Analysis software

(Ingenuity Systems; <http://www.ingenuity.com>) for network analysis. These genes were overlaid onto the global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base. Networks of these genes were then algorithmically generated based on their connectivity for each time point. Network 1 for each time point contained the largest number of genes submitted; a network for each time point is presented in Figure 2. These networks are graphical representations of the molecular relationships between genes or gene products. Genes or gene products are represented as nodes, and the biological relationship between 2 nodes is represented by a line. Dashed lines represent indirect relationships, and solid lines represent direct relationships. Both networks represented in Figure 2 consist primarily of indirect relationships. Intensity of the node color indicates the degree of up- or downregulation in comparison with another group. In Figure 2, the red color indicates increased expression in the SE group compared with the SE + B11 group. Green indicates increased expression in the SE + B11 group compared with the SE group. Pathway figures colored to represent differences among all treatment groups are available in Supplemental Figure 1 (<http://ps.fass.org/content/vol90/issue4>).

Quantitative real-time reverse-transcription PCR was performed on selected genes in the networks generated by Ingenuity Pathway Analysis at each time point. Graphs comparing the microarray analysis and the qRT-PCR analysis of gene expression are presented in Figure 3. Genes evaluated at 12 h include urokinase-type plasminogen activator (*PLAU*), interferon induced with helicase-1 (*IFIH1*), sarcoplasmic/endoplasmic reticulum calcium adenosine triphosphatase 2 (*ATP2A2*), growth arrest-specific 2 (*GAS2*), and ring finger protein 130 (*RNF130*). Among these, *PLAU* was increased in the B11 treatment group only, and *IFIH1* was increased in the SE treatment group only. The *RNF130* was increased in the SE group, but this was not confirmed by qRT-PCR. The *ATP2A2* was moderately but sig-

Table 3. Recovery of *Salmonella* from the cecae of chicks following *S. enterica* serovar Enteritidis (SE) challenge and FloraMax-B11 (B11) treatment¹

Treatment	12 h		24 h	
	No. positive/total no. of samples ² (%)	Colony-forming units/g of cecal content ³	No. positive/total no. of samples (%)	Colony-forming units/g of cecal content
Control	0/10 (0)	ND ⁴	0/10 (0)	ND
B11	0/10 (0)	ND	0/10 (0)	ND
SE	6/10 ^a (60)	4.04 ± 0.61 ^a	10/10 ^a (100)	3.31 ± 0.47 ^a
SE + B11	2/10 ^b (20)	0 ± 0 ^b	5/10 ^b (50)	0.26 ± 0.26 ^b

^{a,b}Means within a time point with differing letters are significantly ($P < 0.05$) different.

¹The SE was obtained from the USDA National Veterinary Services Laboratory (Ames, IA), and the B11 was from Pacific Vet Group USA Inc. (Fayetteville, AR).

²Samples were enriched with tetrathionate broth before plating on xylose lysine deoxycholate (XLD) agar plates containing novobiocin (NO) and naladixic acid (NA) and incubated. The plates were then examined for determination of the presence or absence of SE.

³Samples were diluted in sterile saline and appropriate dilutions were spread plated on XLD agar plates containing NO and NA. Following incubation, the colonies were enumerated to determine the colony-forming units per gram of cecal contents.

⁴ND = not determined.

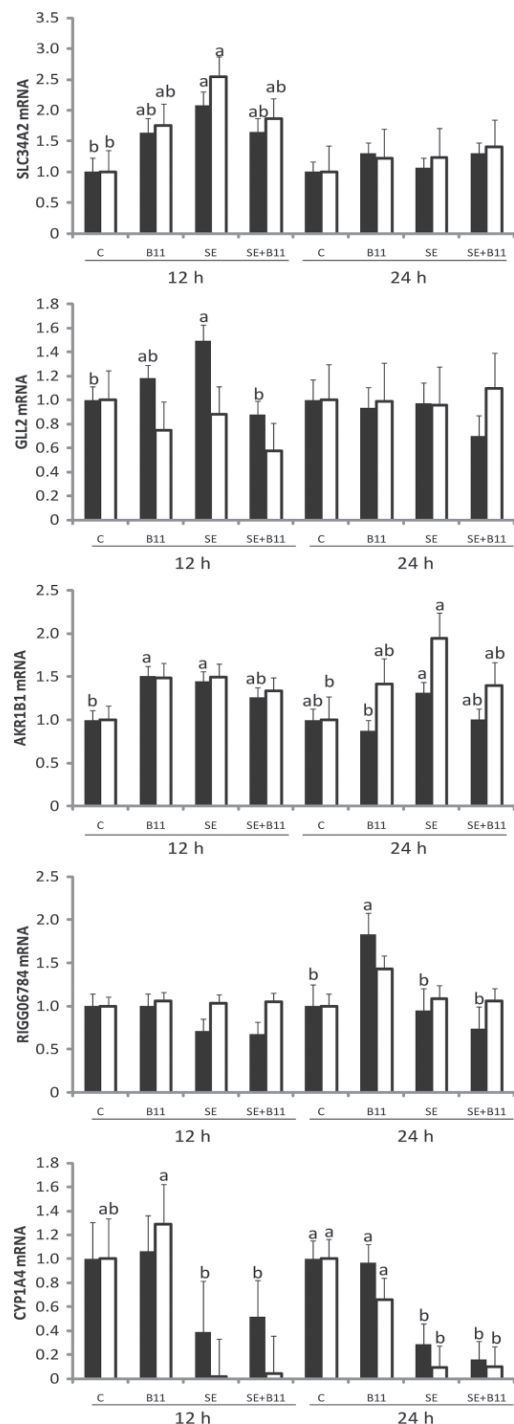


Figure 1. Relative levels of mRNA expression by microarray and quantitative real-time reverse-transcription PCR (qRT-PCR) for selected genes. Data were normalized to the housekeeping gene β -actin (*ACTB*) and are expressed relative to mRNA levels in the control group at each time point (12 and 24 h posttreatment). Treatment groups were designated as follows: control, B11 [not challenged, treated with FloraMax-B11 (Pacific Vet Group USA Inc., Fayetteville, AR)], SE [challenged with *Salmonella enterica* serovar Enteritidis (USDA National Veterinary Services Laboratory, Ames, IA), not treated with B11], and SE + B11 (challenged with SE and treated with B11 one hour postchallenge). The genes evaluated were solute carrier family 34 (sodium phosphate), member 2 (*SLC34A2*), gallinacin-2 (*GLL2*), aldo-keto reductase family 1, member B1 (*AKR1B1*), *Gallus gallus* hypothetical LOC417566 (*RIGG06784*), and cytochrome P450, family 1, subfamily A, polypeptide 4 (*CYP14A4*). Black bars are microarray expression data, and white bars are qRT-PCR expression data. Values within a single time with differing letters (a, b) are significantly different ($P < 0.05$).

nificantly increased in the SE + B11 group only, and *GAS2* was increased by B11 in both the B11 and SE + B11 groups.

At 24 h, the genes evaluated included glutamate-cysteine ligase catalytic subunit (*GCLC*), serotonin 5-HT_{1a} (*HTRA1*), hairy and enhancer of split-1 (*HES1*), cysteine-rich, angiogenic inducer, 61 (*CYR61*), and tumor necrosis factor (TNF) receptor superfamily, member 6 (*FAS*). The *GCLC*, *HES1*, and *CYR61* all were decreased by SE alone. Little change was observed in *HTRA1* and *FAS*.

DISCUSSION

The infection model used in these studies was designed to reflect what occurs when commercial chicks become infected with *Salmonella* in the hatchery and are treated with a probiotic culture on arrival at a farm. The results of the challenge with SE were as expected, with significant reductions of SE recovery in probiotic-treated chicks (Table 3). It should be noted that the colony-forming units recovered from the cecae, as well as the numbers of positive samples, were significantly reduced, indicating that even treated chicks that were positive, or that remained SE infected, harbored lower numbers of SE after treatment compared with untreated chicks.

Microarray analysis of cecal tissues from these chicks revealed that genes were differentially regulated among all treatment groups. Although differences in expression were apparent, the magnitude of differences between treatment groups was not large. However, this is not uncommon. Two other studies evaluating the effects of probiotics likewise observed small differences in levels of gene expression. Shima et al. (2008), who compared gene expression differences in the gastrointestinal tract of mice after administration of different bacterial suspensions, observed 2- to 3-fold differences between significantly different genes, and Brisbin et al. (2008b), who used primary mononuclear cells from chickens incubated with cellular components of *Lactobacillus acidophilus*, observed a maximum of 1.2-fold differences in significantly different genes. Haghghi et al. (2008) evaluated gene expression of selected genes in chicks treated with the probiotic on the day of hatch and challenged with ST at 24 h posttreatment. Gene expression, which was evaluated in the cecal tonsils, a lymphoid tissue at the ileocecal junction, exhibited an increase in interleukin (IL) 12 in response to ST that was maintained at control levels by probiotic treatment at 1 and 5 d postchallenge. Haghghi et al. (2008) also observed that on d 5, ST increased interferon γ (IFN γ) but that probiotic-treated chicks showed reduced levels of IFN γ .

Genes exhibiting the largest differences among groups were confirmed with qRT-PCR (Figure 1). Expression of the chicken Type IIB sodium phosphate cotransporter (*SLC34A2*) gene was increased in chicks after challenge with SE. The *SLC34A2*, as described previously in the

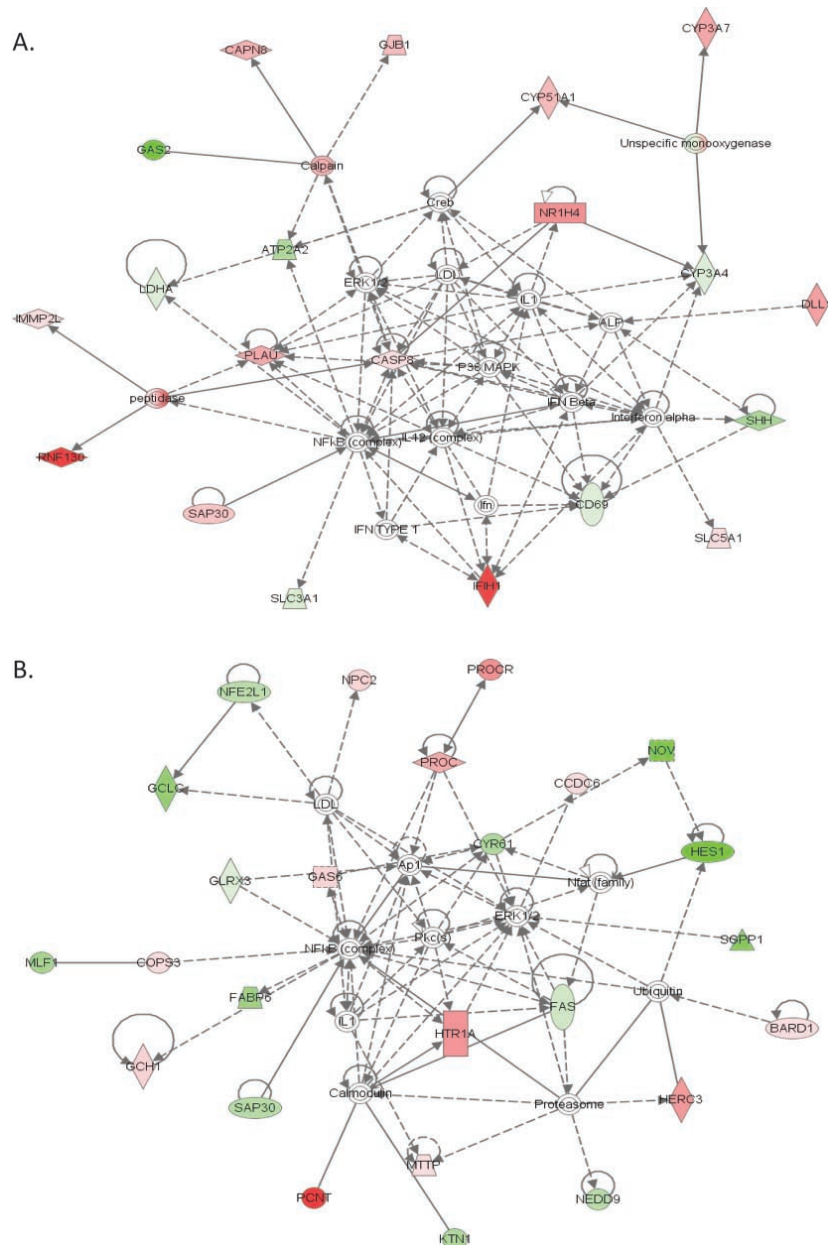


Figure 2. Gene networks proposed by Ingenuity Pathway Analysis software (<http://www.ingenuity.com>) for genes differentially expressed ($P < 0.05$, fold difference ≥ 1.2) at 12 h (A) and 24 h (B) post-treatment. The intensity of red and green shaded genes indicates differences in expression levels between the SE treatment [challenged with *Salmonella enterica* serovar Enteritidis (USDA National Veterinary Services Laboratory, Ames, IA), not treated with B11 (FloraMax-B11, Pacific Vet Group USA Inc., Fayetteville, AR), and the SE + B11 treatment (challenged with SE and treated with B11 one hour postchallenge)]. Red indicates increased expression in the SE treatment group as compared with the SE + B11 treatment group. Green indicates increased expression in the SE + B11 treatment group as compared with the SE treatment group. White genes were inferred by the software, and expression levels were not determined or were not significant in this experiment. *CAPN8* = calpain 8; *GJB1* = gap junction protein, $\beta 1$; *CYP3A7* = cytochrome P450, family 3, subfamily A, polypeptide 7; *CYP51A1* = cytochrome P450, family 51, subfamily A, polypeptide 1; *GAS2* = growth arrest-specific 2; *Creb* = cAMP response element-binding; *NR1H4* = nuclear receptor subfamily 1, group H, member 4; *ATP2A2* = sarcoplasmic/endoplasmic reticulum calcium adenosine triphosphatase 2; *CYP3A4* = cytochrome P450, family 3, subfamily A, polypeptide 4; *ERK12* = extracellular-signal-regulated kinases 1/2; *LDL* = low-density lipoprotein complex; *LDHA* = lactate dehydrogenase A; *IL1* = interleukin 1; *DLL1* = delta-like 1; *IMMP2L* = inner mitochondrial membrane peptidase-like; *ALP* = alkaline phosphatase; *PLAU* = urokinase-type plasminogen activator; *CASP8* = caspase 8, apoptosis-related cysteine peptidase; *P36MAPK* = p36 mitogen-activated protein kinase; *IFN β* = interferon β ; *SHH* = sonic hedgehog; *Nf κ B* = nuclear factor kappa B; *IL12* = interleukin 12; *RNF130* = ring finger protein 130; *Ifn* = interferon; *CD69* = p60, early T-cell activation antigen; *SAP30* = Sin3A-associated protein; *IFN Type 1* = interferon type 1; *SLC3A1* = solute carrier family 3 (cystine, dibasic and neutral amino acid transporters), member 1; *PROCR* = protein C receptor, endothelial; *NPC2* = Niemann-Pick disease, type C2; *NFE2L1* = nuclear factor (erythroid-derived 2)-like 1; *NOV* = nephroblastoma overexpressed gene; *PROC* = protein C; *CCDC6* = coiled-coil domain containing 6; *GCLC* = glutamate-cysteine ligase catalytic subunit; *CYR61* = cysteine-rich, angiogenic inducer, 61; *HES1* = hairy and enhancer of split-1; *Ap1* = activator protein 1; *GLRX3* = glutaredoxin; *GAS6* = growth arrest-specific 6; *Nfat* = nuclear factor of activated T-cells; *SGPP1* = sphingosine-1-phosphate phosphatase 1; *Pkc(s)* = protein kinase c; *MLF1* = myeloid leukemia factor 1; *COP38* = COP9 constitutive photomorphogenic homolog subunit 8; *FABP6* = fatty acid binding protein 6, ileal; *FAS* = tumor necrosis factor receptor superfamily, member 6; *IL1* = interleukin 1; *HTR1A* = serotonin 5-HT_{1A}; *BARD1* = BRCA1 associated RING domain 1; *GCH1* = GTP cyclohydrolase 1; *HERC3* = hect domain and RLD 3; *MTTP* = microsomal triglyceride transfer protein; *PCNT* = pericentrin; *NEDD9* = neural precursor cell expressed, developmentally down-regulated 9; *KTN1* = kinctin 1.

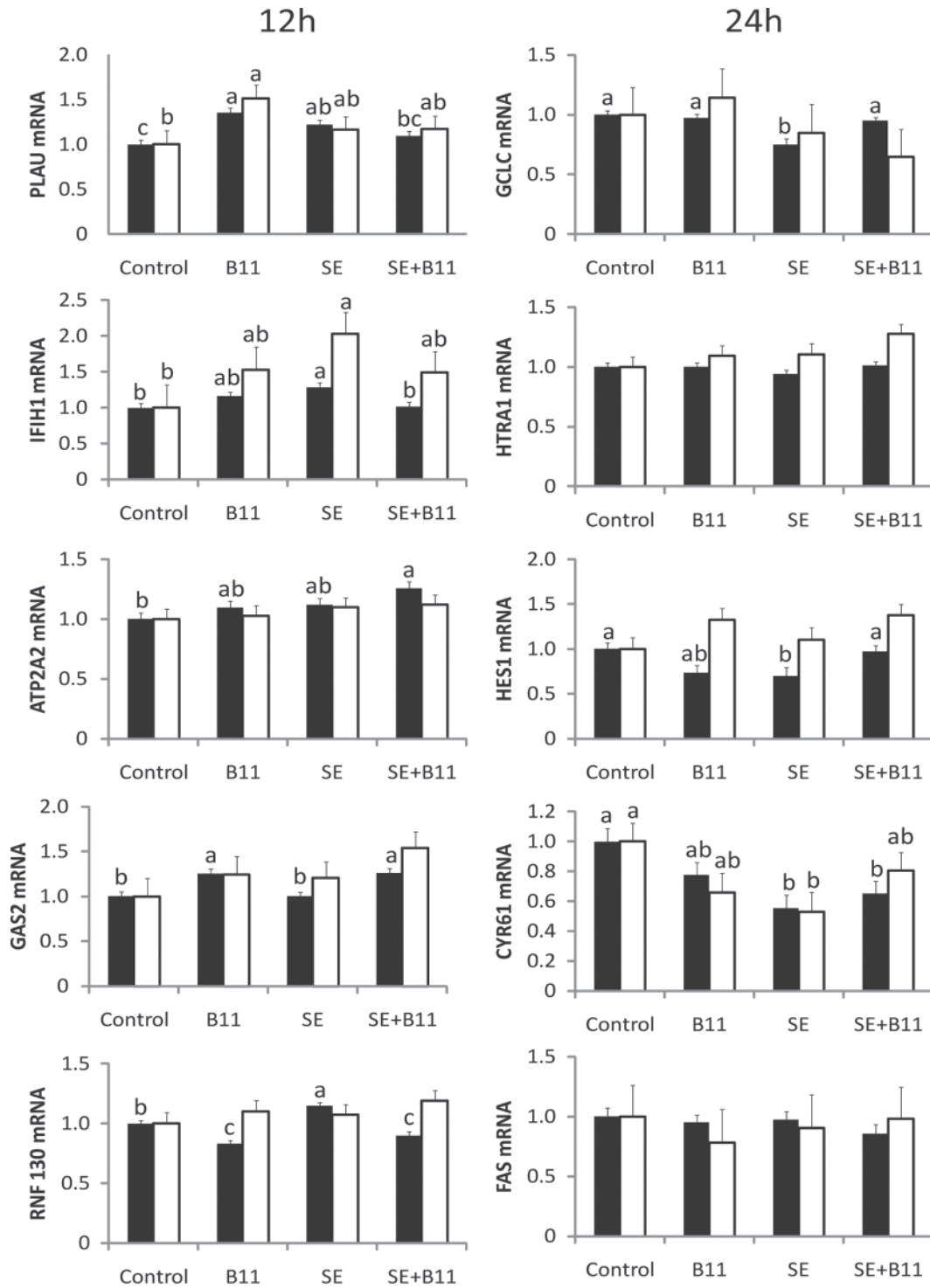


Figure 3. Relative levels of mRNA expression by microarray and quantitative real-time reverse-transcription PCR (qRT-PCR) for selected genes included in the predicted gene network at 12 and 24 h. Data were normalized to the housekeeping gene β -actin (*ACTB*) and are expressed relative to mRNA levels in the control group at each time point (12 and 24 h posttreatment). Treatment groups are designated as follows: control, B11 [not challenged, treated with FloraMax-B11 (Pacific Vet Group USA Inc., Fayetteville, AR)], SE [challenged with *Salmonella enterica* serovar Enteritidis (USDA National Veterinary Services Laboratory, Ames, IA), not treated with B11], and SE + B11 (challenged with SE and treated with B11 one hour postchallenge). Genes evaluated at 12 h were urokinase-type plasminogen activator (*PLAU*), interferon induced with helicase-1 (*IFIH1*), sarcoplasmic/endoplasmic reticulum calcium adenosine triphosphatase 2 (*ATP2A2*), growth arrest-specific 2 (*GAS2*), and ring finger protein 130 (*RNF130*). Genes evaluated at 24 h were glutamate-cysteine ligase catalytic subunit (*GCLC*), serotonin 5-HT_{1a} (*HTRA1*), hairy and enhancer of split-1 (*HES1*), cysteine-rich, angiogenic inducer, 61 (*CYR61*), and tumor necrosis factor receptor superfamily, member 6 (*FAS*). Black bars are microarray expression data, and white bars are qRT-PCR expression data. Values within a single time with differing letters (a, b) are significantly different ($P < 0.05$).

small intestine, was increased in response to reduced phosphorus in the diet of chicks (Yan et al., 2007). The significance of the transient upregulation in response to SE is not clear at present. We likewise observed a similar response of the β -defensin gene gallinacin 2 (*GLL2*), which increased at 12 h in the presence of SE, but not SE + B11. The β -defensins are antimicrobial peptides expressed by heterophils and intestinal epithelial cells in chickens (Derache et al., 2009). Derache et al. (2009) also observed an increase in *GLL2* expression in embryonic primary cecal cell cultures in response to killed SE, and Akbari et al. (2008) observed similar expression data for *GLL2* in the cecal tonsils of chickens treated with probiotics and challenged with ST. Taken together, it appears that *Salmonella* spp. increase expression of *GLL2* and that *Lactobacillus* generally do not increase *GLL2*. The reason for the upregulation of *GLL2* in the presence of SE is not known, but may be postulated as a protective response to that specific pathogen. Despite the increase in expression of *GLL2* by SE at 12 h, the SE infection was still increasing at 24 h, when *GLL2* expression was not different from that of the controls, suggesting that the ability of *GLL2* to inhibit SE in vivo is limited.

We also observed an increase in *AKR1B1* at 24 h in response to SE. This gene is responsible for mediating cytotoxic signals by binding to nuclear factor kappa B (NF κ B) and activator protein 1. The *AKR1B1* is induced by many things, including lipopolysaccharide (LPS), which is present on the surface of *Salmonella* and other gram-negative bacteria. Ramana and Srivastava (2006) examined the function of *AKR1B1* by incubating mouse macrophages with inhibitors of *AKR1B1* followed by stimulation of the macrophages with LPS. Treatment with *AKR1B1* inhibitors resulted in inhibition of binding to NF κ B and activator protein 1, reduced cytokine production, prostaglandin E₂ production, cyclooxygenase-2 stimulation, and nitric oxide production. Potentially, B11 is inhibiting *AKR1B1* expression, resulting in less inflammation and fewer potential cells to harbor SE. Alternatively, the lower levels of SE in the SE + B11 group may be not be sufficient to induce greater gene expression of *AKR1B1*.

One gene was significantly increased only by B11 at the 24-h time point. This gene, *RIGG06784*, is not characterized, but has some homology with transmembrane protein 199, indicating that it is possibly expressed on the surface of the epithelial cells and induced by B11 treatment. We also observed significantly decreased expression of *CYP1A4* at 12 and 24 h in the SE and SE + B11 treatment groups. This is interesting because *CYP1A4* expression was decreased despite the fact that the SE + B11 treatment groups had significantly less SE infection, with most chicks completely uncolonized. Cytochrome P450, family 1, subfamily A, polypeptide 4 has not been previously associated with responses to SE, and it is primarily associated with responses to environmental toxins such as dioxin-like compounds (Head and Kennedy, 2007).

We chose to further evaluate genes that were significantly different ($P < 0.05$) and with a minimum of 1.2-fold differences in expression levels across treatment groups within a single time point (12 or 24 h) by using Ingenuity Pathway Analysis software. The gene network that included the largest subset of candidate genes from the microarray analysis at both 12 and 24 h included NF κ B as a major node, although the surrounding genes in each network were different at each time. Nuclear factor kappa B is a transcription factor that, when activated in the cytosol, translocates to the nucleus, where it initiates transcription of genes necessary for inflammation and immune responsiveness. Nuclear factor kappa B comprises up to 5 subunits, which form homodimers and heterodimers; the components of the dimers determine specificity regarding the genes transcribed. Nuclear factor kappa B is bound in the cytosol by inhibitor of κ B (I κ B), and on activation, I κ B is phosphorylated and then ubiquitinated, freeing NF κ B to translocate to the nucleus. (Eaves-Pyles et al., 1999, Spehlmann and Eckmann, 2009). Ablation of subunits of the I κ B kinase complex responsible for activation of NF κ B in the intestinal epithelial cells of mice resulted in spontaneous colitis, intestinal inflammation, and invasion of commensal bacteria into the mucosal layer of cells, indicating that NF κ B is also necessary for maintenance of an intact barrier in the intestinal tract (Pasparakis, 2008).

Bacteria are known to activate NF κ B in numerous ways. It has been observed that *Salmonella* activates NF κ B and proinflammatory gene transcription by adhesion, but invasion is not necessary (Eaves-Pyles et al., 1999). Both secretion of the effector protein *Salmonella* outer protein E and activation of Toll-like receptor 5 by flagellin are mechanisms that can activate NF κ B without internalization of the SE by the host cell. These observations explain the phenomenon that intestinal *Salmonella* infections generally result in invasion of only approximately 30% of cells, yet NF κ B activation is observed in many more cells (Tallant et al., 2004). On the other hand, ST effector proteins secreted by ST in the cell culture are capable of inhibiting gene expression induced by NF κ B and possibly delaying macrophage toxicity (Haraga and Miller, 2003; LeNegrata et al., 2008). Remarkably, Neish et al. (2000) showed that nonvirulent *Salmonella* strains were capable of inhibiting proinflammatory gene expression by blocking degradation, which prevents the nuclear translocation of NF κ B, suggesting that activation or inhibition of NF κ B is a condition of pathogenicity. Therefore, *Salmonella* spp. are capable of both activating and inhibiting this critical transcription factor. Importantly, regulation of NF κ B has also been reported after treatment with probiotics. Miettinen et al. (2000) previously observed activation of NF κ B by both *Lactobacillus* and *Streptococcus*, which induced both IL-6 and TNF α , indicating that NF κ B responds to both commensal and pathogenic gram-positive bacterial species. Additionally, expression of NF κ B subunits was upregulated in the duodenal

mucosa of human patients 6 h after consumption of dead *Lactobacillus plantarum* cultures (vanBaarlen et al., 2009). Mice receiving *Bacillus infantis* as a probiotic in the drinking water for 3 wk before challenge with ST had reduced NF κ B activation in response to challenge compared with mice that did not receive the probiotic treatment (O'Mahony et al., 2008).

At 12 h, genes that were differentially regulated in the network and that interacted with NF κ B included *IFIH1*, *PLAU*, and *ATP2A2*. Interferon induced with helicase-1, also known as melanoma differentiation associated gene-5 (*MDA5*), is a gene whose product is a cytoplasmic viral RNA sensor. The *IFIH1* contains 2 N-terminal caspase recruitment domains, which will activate downstream signaling caspases, resulting in activation of NF κ B and other transcriptional factors (Unterholzner and Bowie, 2008). Although *IFIH1* and the similar helicase retinoic acid inducible gene-I (*RIG-I*) are thought to recognize double-stranded RNA, there are differing specificities between the 2, indicating that unknown factors may also play a role in the recognition of pathogens (Unterholzner and Bowie, 2008). Expression of this gene was increased in SE-challenged chicks at 12 h. There are no known reports of this gene being associated with *Salmonella* infection. Potentially, the activation of *IFIH1* allows for initiation of proinflammatory genes through NF κ B activation. Although *IFIH1* is previously understood to recognize viral RNA, the ability of *Salmonella* to increase the expression of this gene may provide further understanding of the function of *IFIH1*. The *PLAU* encodes a serine protease that is involved in degradation of the extracellular matrix. Studies in a breast cancer cell line revealed that inhibition of *PLAU* resulted in apoptosis (Ma et al., 2001). Therefore, increased expression of *PLAU* in the presence of B11 (Figure 3) compared with the control may indicate that B11 promotes cell growth and survival in the cecae. The *ATP2A2* primarily functions as a calcium pump in muscle cells, and its expression was moderately but significantly increased in chicks treated with SE + B11. In experiments using rat myocytes, *ATP2A2* was shown to increase the transcription of NF κ B (Shah et al., 2005).

Another differentially regulated gene at 12 h was *GAS2*, which was elevated in expression in both the B11 and SE + B11 treatment groups at 12 h. The *GAS2* binds milli-calpain, increasing the stability of p53, and ultimately increasing the susceptibility of cells to apoptosis (Benetti et al., 2001). It is possible that apoptosis of SE-infected cells is required to curtail SE infection, and increased expression of *GAS2* in B11-treated chicks allowed increased apoptosis.

The *CYR61* was significantly downregulated in the cecae of chicks after SE infection. However, in both treatment groups receiving B11 (B11 and SE + B11), *CYR61* was not expressed at significantly lower levels than in the control. The *CYR61* is one member of the CCN family [i.e., connective tissue growth factor, *CYR61*, and nephroblastoma overexpressed gene] of ex-

tracellular matrix-associated proteins, each containing 4 cysteine-rich conserved domains. The CCN proteins bind heparin sulfate proteoglycans and specific integrins that determine the specificity of the effector functions (Chen and Lau, 2009). Importantly, *CYR61* is associated with induction of 2 distinct apoptotic pathways. The first is through synergism with Fas ligand (**FasL**). The FasL produced by lymphocytes binds the FAS receptor, which is produced by many cell types, to mediate apoptosis. The *CYR61* binds the integrin $\alpha_6\beta_1$ and heparan sulfate proteoglycans, in the presence of FasL, leading to accumulation of reactive oxygen species and eventually apoptosis (Juric et al., 2009). The *FAS* was shown previously to be downregulated in response to SE in cecae (Berndt et al., 2007); however, in the present study, *FAS* was not consistently regulated in response to treatment. Alternatively, *CYR61* is also capable of unmasking the apoptotic potential of TNF α . Tumor necrosis factor α is responsible for promoting cell proliferation through NF κ B signaling, and is generally capable of inducing apoptosis only when NF κ B signaling or protein synthesis is blocked. However, CCN1 bound to integrin $\alpha_6\beta_1$ in the presence of TNF α likewise leads to increased intracellular reactive oxygen species, which allows TNF α -induced apoptosis without the blocking of NF κ B activation or protein synthesis (Chen et al., 2007). Importantly, *CYR61* was downregulated in the cecae of SE-challenged chicks at 24 h postchallenge, which may be a survival mechanism of the SE to avoid elimination through apoptosis of the SE-infected cell. Differential expression of *CYR61* in response to SE infection has not been reported previously and may prove to be a mechanism by which SE promotes intracellular survival by avoiding apoptosis.

In this experiment, we did not observe the regulation of genes involved in an innate immune response as we expected. Previous studies reporting expression of genes measured in the cecal tonsils (Haghighi et al., 2008), spleen (Zhou and Lamont, 2007), heterophils (Chiang et al., 2008), or macrophages (Zhang et al., 2008) have revealed robust increases in inflammatory cytokines and other innate immune genes following *Salmonella* challenge. We postulate that the much lower density of immune cells in the whole cecal tissue, compared with the previously studied tissues or cells, may dilute out the observable changes in immune gene expression at these time points. One study performed by Berndt et al. (2007) in specific-pathogen-free Leghorn chicks did report an increase in IL-8, IL-12, IL-7R α , and LPS-induced TNF in the cecal tissue at 1 d after challenge with SE. However, the present data were collected from broiler chicks, which, in previous infection studies using a virulent strain of phage type 4 SE, demonstrated less morbidity and mortality as compared with those of Leghorn chicks (Gast and Benson, 1995). This perhaps explains the lack of increased expression of immune system genes in the present experiment.

Previous microarray studies have also been performed following application of probiotics to cultured

cells. Cultured colonocytes (Caco-2 cell line) incubated in the presence of a commensal *Escherichia coli* or *L. plantarum* resulted in different gene expression profiles (Panigrahi et al., 2007). Shima et al. (2008) compared with gene expression from the intestinal epithelial cells of mice from different regions of the gastrointestinal tract after administration of *Lactobacillus casei*, *Bifidobacterium breve*, segmented filamentous bacteria, or a fecal suspension. They likewise observed that differential gene expression was bacterial strain dependent. Brisbin et al. (2008a) evaluated the effects of *L. acidophilus* cellular components on mononuclear cells isolated from the spleen or cecal tonsils of mature Leghorn chickens using a low-density microarray, and observed that the mononuclear cells responded primarily to DNA rather than to cell wall components. Haghighi et al. (2008) evaluated the gene expression of selected genes in chicks treated with a probiotic on the day of hatch and challenged with ST 24 h posttreatment. Gene expression was evaluated in the cecal tonsils, a lymphoid tissue at the ileocecal junction, which showed an increase in IL-12 in response to ST that was maintained at control levels by probiotic treatment at 1 and 5 d postchallenge. On d 5, it was also observed that ST increased IFN γ , but chicks treated with probiotic showed reduced levels of IFN γ . We did not observe similar responses in this study; however, our samples were obtained at earlier time points and in a different tissue (whole caecae). To our knowledge, the present data are the first report of global gene expression changes in chicks in response to both SE and probiotic treatment.

Importantly, probiotics have previously been reported to regulate apoptosis in vitro and in vivo. Lin et al. (2008) and Mennigen et al. (2009) both reported increased resistance to apoptosis when using murine models after administration of the probiotics *Lactobacillus GG* or the VSL#3 mixed culture, respectively. However, Iyer et al. (2008) observed an increase in apoptosis in myeloid leukemia-derived cells, mediated through NF κ B activation after treatment with *Lactobacillus reuteri*. These data indicate that probiotic bacteria are indeed capable of regulating apoptosis, and that the manner in which they do so is apparently different depending on the strain or strains of probiotic bacteria or cultures administered.

In this experiment, we evaluated the gene expression profiles in cecal tissue of chicks challenged with SE and treated or not treated with B11 at 12 and 24 h posttreatment. We observed changes in global gene expression in all groups, which were potentially related to activation, but not expression, of NF κ B. Based on the gene expression profiles of genes present in the postulated networks, we propose that differences in expression of some genes, such as *GAS2* at 12 h, and *CYR61* at 24 h, facilitate an increase in apoptosis of SE-infected cells in the presence of the B11 probiotic culture, which results in a decrease in the numbers of colonized bacteria in the caecae of chicks.

ACKNOWLEDGMENTS

This work was supported by grant number 2008-32201-04667 from USDA-Cooperative State Research, Education, and Extension Service (Washington, DC).

REFERENCES

- Akbari, M. R., H. R. Haghighi, J. R. Chambers, J. Brisbin, L. R. Read, and S. Sharif. 2008. Expression of antimicrobial peptides in cecal tonsils of chickens treated with probiotics and infected with *Salmonella enterica* serovar Typhimurium. *Clin. Vaccine Immunol.* 15:1689–1693.
- Bailey, J. S., N. A. Cox, S. E. Craven, and D. E. Cosby. 2002. Serotype tracking of *Salmonella* through integrated broiler chicken operations. *J. Food Prot.* 65:742–745.
- Bailey, J. S., N. J. Stern, and N. A. Cox. 2000. Commercial field trial evaluation of mucosal starter culture to reduce *Salmonella* incidence in processed broiler carcasses. *J. Food Prot.* 63:867–870.
- Benetti, R., G. Del Sal, M. Monte, G. Paroni, C. Brancolini, and C. Schneider. 2001. The death substrate Gas2 binds m-calpain and increases susceptibility to p53-dependent apoptosis. *EMBO J.* 20:2702–2714.
- Berndt, A., A. Wilhelm, C. Jugert, J. Pieper, K. Sachse, and U. Methner. 2007. Chicken cecum immune response to *Salmonella enterica* serovars of different levels of invasiveness. *Infect. Immun.* 75:5993–6007.
- Brisbin, J. T., J. Gong, and S. Sharif. 2008a. Interactions between commensal bacteria and the gut-associated immune system of chickens. *Anim. Health Res. Rev.* 9:101–110.
- Brisbin, J. T., H. Zhou, J. Gong, P. Babour, M. R. Akbari, H. R. Haghighi, H. Yu, A. Clarke, A. J. Sarson, and S. Sharif. 2008b. Gene expression profiling of chicken lymphoid cells after treatment with *Lactobacillus acidophilus* cellular components. *Dev. Comp. Immunol.* 32:563–574.
- Callaway, T. R., T. S. Edrington, R. C. Anderson, J. A. Byrd, and D. J. Nisbet. 2008. Gastrointestinal microbial ecology and the safety of our food supply as related to *Salmonella*. *J. Anim. Sci.* 86:E163–E172.
- Centers for Disease Control and Prevention. 2009. Preliminary Food-Net Data on the incidence of infection with pathogens transmitted commonly through food—10 States, 2008. *MMWR Morb. Mortal. Wkly. Rep.* 58:333–337.
- Chen, C. C., and L. F. Lau. 2009. Functions and mechanisms of action of CCN matricellular proteins. *Int. J. Biochem. Cell Biol.* 41:771–783.
- Chen, C. C., J. L. Young, R. I. Monzon, N. Chen, V. Todorovic, and L. F. Lau. 2007. Cytotoxicity of TNF-alpha is regulated by integrin-mediated matrix signaling. *EMBO J.* 26:1257–1267.
- Chiang, H. I., C. L. Swaggerty, M. H. Kogut, S. E. Dowd, X. Li, I. Y. Pevzner, and H. Zhou. 2008. Gene expression profiling in chicken heterophils with *Salmonella enteritidis* stimulation using a chicken 44k Agilent microarray. *BMC Genomics* 9:526.
- Corrier, D. E., D. J. Nisbet, J. A. Byrd III, B. M. Hargis, N. K. Keith, M. Peterson, and J. R. Deloach. 1998. Dosage titration of a characterized competitive exclusion culture to inhibit *Salmonella* colonization in broiler chickens during growout. *J. Food Prot.* 61:796–801.
- Derache, C., E. Esnault, C. Bonsergent, Y. Le Vern, P. Quééré, and A. Lalmanach. 2009. Differential modulation of β -defensin gene expression by *Salmonella* Enteritidis in intestinal epithelial cells from resistant and susceptible chicken inbred lines. *Dev. Comp. Immunol.* 33:959–966.
- Eaves-Pyles, T., C. Szabo, and A. L. Salzman. 1999. Bacterial invasion is not required for activation of NF- κ B in enterocytes. *Infect. Immun.* 67:800–804.
- Ellestad, L. E., W. Carre, M. Muchow, S. A. Jenkins, X. Wang, L. A. Cogburn, and T. E. Porter. 2006. Gene expression profiling during cellular differentiation in the embryonic pituitary gland using cDNA microarrays. *Physiol. Genomics* 25:414–425.
- Gast, R. K., and S. T. Benson. 1995. The comparative virulence for chicks of *Salmonella enteritidis* phage type 4 isolates and isolates

- of phage types commonly found in poultry in the United States. *Avian Dis.* 39:567–574.
- Haghighi, H. R., M. F. Abdul-Careem, R. A. Dara, J. R. Chambers, and S. Sharif. 2008. Cytokine gene expression in chicken cecal tonsils following treatment with probiotics and *Salmonella* infection. *Vet. Microbiol.* 126:225–233.
- Haraga, A., and S. I. Miller. 2003. A *Salmonella enterica* serovar Typhimurium translocated leucine-rich repeat effector protein inhibits NF- κ B dependent gene expression. *Infect. Immun.* 71:4052–4058.
- Hargis, B. M., D. J. Caldwell, and J. A. Byrd. 2001. *Microbial Pathogens of Poultry: Live Bird Considerations*. CRC Press LLC, Boca Raton, FL.
- Head, J. A., and S. W. Kennedy. 2007. Differential expression, induction, and stability of *CYP1A4* and *CYP1A5* mRNA in chicken and herring gull embryo hepatocytes. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 145:617–624.
- Higgins, J. P., S. E. Higgins, J. L. Vicente, A. D. Wolfenden, G. Tellez, and B. M. Hargis. 2007. Temporal effects of lactic acid bacteria probiotic culture on *Salmonella* in neonatal broilers. *Poult. Sci.* 86:1662–1666.
- Higgins, J. P., S. E. Higgins, A. D. Wolfenden, S. N. Henderson, A. Torres-Rodriguez, J. L. Vicente, B. M. Hargis, and G. Tellez. 2010. Effect of lactic acid bacteria probiotic culture treatment timing on *Salmonella* Enteritidis in neonatal broilers. *Poult. Sci.* 89:243–247.
- Higgins, S. E., J. P. Higgins, A. D. Wolfenden, S. N. Henderson, A. Torres-Rodriguez, G. Tellez, and B. M. Hargis. 2008. Evaluation of a *Lactobacillus*-based probiotic culture for the reduction of *Salmonella* Enteritidis in neonatal chicks. *Poult. Sci.* 87:27–31.
- Higgins, S. E., A. Torres-Rodriguez, J. L. Vicente, C. D. Sartor, C. M. Pixley, G. M. Nava, G. Tellez, J. T. Barton, and B. M. Hargis. 2005. Evaluation of intervention strategies for idiopathic diarrhea in commercial turkey brooding houses. *J. Appl. Poult. Res.* 14:345–348.
- Iyer, C., A. Kusters, G. Sethi, A. B. Kunnumakkara, B. B. Aggarwal, and J. Versalovic. 2008. Probiotic *Lactobacillus reuteri* promotes TNF-induced apoptosis in human myeloid leukemia-derived cells by modulation of NF- κ B and MAPK signalling. *Cell. Microbiol.* 10:1442–1452.
- Juric, V., C. Chen, and L. F. Lau. 2009. Fas-mediated apoptosis is regulated by the extracellular protein CCN1 (CYR61) in vitro and in vivo. *Mol. Cell. Biol.* 29:3266–3279.
- Kimura, A. C., V. Reddy, R. Marcus, P. R. Cieslak, J. C. Mohle-Boetani, H. D. Kassenborg, S. D. Seler, F. P. Hardnett, T. Barrett, and D. L. Swerdlow. 2004. Chicken consumption is a newly identified risk factor for sporadic *Salmonella enterica* serotype Enteritidis infections in the United States: A case-control study in FoodNet site. *Clin. Infect. Dis.* 38(Suppl. 3):S244–S252.
- Le Negrate, G., B. Faustin, K. Welsh, M. Loeffler, M. Krajewska, P. Hasegawa, S. Mukherjee, K. Orth, S. Krajewske, A. Godzik, D. G. Guiney, and J. C. Reed. 2008. *Salmonella* secreted factor L deubiquitinase of *Salmonella typhimurium* inhibits NF- κ B, suppresses I κ B α ubiquitination and modulates innate immune responses. *J. Immunol.* 180:5045–5056.
- Lin, P. W., T. R. Nasr, A. J. Berardinelli, A. Kumar, and A. S. Neish. 2008. The probiotic *Lactobacillus* GG may augment intestinal host defense by regulating apoptosis and promoting cytoprotective responses in the developing murine gut. *Pediatr. Res.* 64:511–516.
- Ma, Z., D. J. Webb, M. Jo, and S. L. Gonias. 2001. Endogenously produced urokinase-type plasminogen activator is a major determinant of the basal level of activated ERK/MAP kinase and prevents apoptosis in MDA-MB-231 breast cancer cells. *J. Cell Sci.* 114:3387–3396.
- Marcus, R., J. K. Varma, C. Medus, E. J. Boothe, B. J. Anderson, T. Crume, K. E. Fullerton, M. R. Moore, P. L. White, E. Lyszkowicz, A. C. Voetsch, and F. J. Angulo. 2007. Re-assessment of risk factors for sporadic *Salmonella* serotype Enteritidis infections: A case-control study in five FoodNet sites, 2002–2003. *Epidemiol. Infect.* 135:84–92.
- McCrea, B. A., K. S. Macklin, R. A. Norton, J. B. Hess, and S. F. Bilgili. 2006. A longitudinal study of *Salmonella* and *Campylobacter jejuni* isolates from day of hatch through processing by automated ribotyping. *J. Food Prot.* 69:2908–2914.
- Mennigen, R., K. Nolte, E. Rijcken, M. Utech, B. Loeffler, N. Senninger, and M. Bruewer. 2009. Probiotic mixture VSL#3 protects the epithelial barrier by maintaining tight junction protein expression and preventing apoptosis in a murine model of colitis. *Am. J. Physiol. Gastrointest. Liver Physiol.* 296:G1140–G1149.
- Miettinen, M., A. Lehtonen, I. Julkunen, and S. Matikainen. 2000. Lactobacilli and streptococci activate NF- κ B and STAT signaling pathways in human macrophages. *J. Immunol.* 164:3733–3740.
- Neish, A. S., A. T. Gewirtz, H. Zeng, A. N. Young, M. E. Hobert, V. Karmali, A. S. Rao, and J. L. Madara. 2000. Prokaryotic regulation of epithelial responses by inhibition of I κ B- α ubiquitination. *Science* 289:1560–1563.
- O'Mahony, C., P. Scully, D. O'Mahoney, S. Murphy, F. O'Brien, A. Lyons, G. Sherlock, J. MacSharry, B. Keily, F. Shanahan, and L. O'Mahoney. 2008. Commensal-induced regulatory T cells mediate protection against pathogen-stimulated NF- κ B activation. *PLoS Pathog.* 4:e1000112.
- Panigrahi, P., G. T. Braileanu, H. Chen, and O. C. Stine. 2007. Probiotic bacteria change *Escherichia coli*-induced gene expression in cultured colonocytes: Implication in intestinal pathophysiology. *World J. Gastroenterol.* 13:6370–6378.
- Pasparakis, M. 2008. IKK/NF- κ B signaling in intestinal epithelial cells controls immune homeostasis in the gut. *Mucosal Immunol.* 1:S54–S57.
- Patterson, J. A., and K. M. Burkholder. 2003. Application of prebiotics and probiotics in poultry. *Poult. Sci.* 82:627–631.
- Phillips, J., and J. H. Eberwine. 1996. Antisense RNA amplification: A linear amplification method for analyzing the mRNA population from single living cells. *Methods* 10:283–288.
- Ramana, K. V., and S. K. Srivastava. 2006. Mediation of aldose reductase in lipopolysaccharide-induced inflammatory signals in mouse peritoneal macrophages. *Cytokine* 36:115–122.
- Rantala, M., and E. Nurmi. 1973. Prevention of the growth of *Salmonella infantis* in chicks by the flora of the alimentary tract of chickens. *Br. Poult. Sci.* 14:627–630.
- Revolledo, L., A. J. P. Ferreira, and G. C. Mead. 2006. Prospects in *Salmonella* control: Competitive exclusion, probiotics, and enhancement of avian intestinal immunity. *J. Appl. Poult. Res.* 15:341–351.
- Rozen, S., and H. J. Skaletsky. 2000. *Primer3 on the WWW for General Users and for Biologist Programmers*. Humana Press, Totowa, NJ.
- Saeed, A. I., V. Sharov, J. White, J. Li, W. Liang, N. Bhagabati, J. Braisted, M. Klapa, T. Currier, M. Thiagarajan, A. Sturm, M. Snuffin, A. Rezantsev, D. Popov, A. Ryltsov, E. Kostukovich, I. Borisovsky, Z. Liu, A. Vinsavich, V. Trush, and J. Quackenbush. 2003. TM4: A free, open-source system for microarray data management and analysis. *Biotechniques* 34:374–378.
- Shah, R. D., F. Gonzales, E. Golez, D. Augustin, S. Caudillo, A. Abbott, J. Morello, P. M. McDonough, P. J. Paolini, and H. E. Shubaita. 2005. The antidiabetic agent rosiglitazone upregulates SERCA2 and enhances TNF- α and LPS-induced NF- κ B-dependent transcription and TNF- α -induced IL-6 secretion in ventricular myocytes. *Cell. Physiol. Biochem.* 15:41–50.
- Shima, T., K. Fukushima, H. Setoyama, A. Imaoka, S. Matsumoto, T. Hara, K. Suda, and Y. Umesake. 2008. Differential effects of two probiotic strains with different bacteriological properties on intestinal gene expression, with special reference to indigenous bacteria. *FEMS Immunol. Med. Microbiol.* 52:69–77.
- Simon, R., M. D. Radmacher, and K. Dobbin. 2002. Design of studies using DNA microarrays. *Genet. Epidemiol.* 23:21–36.
- Spehlmann, M. E., and L. Eckmann. 2009. Nuclear factor- κ B in intestinal protection and destruction. *Curr. Opin. Gastroenterol.* 25:92–99.
- Tallant, T., A. Deb, N. Kar, J. Lupica, M. J. deVeer, and J. A. DiDonato. 2004. Flagellin acting via TLR5 is the major activator of key signaling pathways leading to NF- κ B and proinflammatory gene program activation in intestinal epithelial cells. *BMC Microbiol.* 4:33.
- Unterholzner, L., and A. G. Bowie. 2008. The interplay between viruses and innate immune signaling: Recent insights and therapeutic opportunities. *Biochem. Pharmacol.* 75:589–602.

- van Baarlen, P., F. J. Troost, S. vanHemert, C. van der Meer, W. M. de Vos, P. J. de Groot, G. J. E. J. Hooiveld, R. M. Brummer, and M. Kleerebezem. 2009. Differential NF- κ B pathways induction by *Lactobacillus plantarum* in the duodenum of healthy humans correlating with immune tolerance. *Proc. Natl. Acad. Sci. USA* 106:2371–2376.
- Vicente, J. L., A. Torres-Rodriguez, S. E. Higgins, X. Hernández-Velasco, C. Pixley, G. Tellez, A. M. Donoghue, and B. M. Hargis. 2008. Effect of a selected *Lactobacillus* species-based probiotic on *Salmonella enterica* serovar Enteritidis-infected broiler chicks. *Avian Dis.* 52:143–146.
- Vicente, J. L., A. D. Wolfenden, A. Torres-Rodriguez, X. Hernandez, S. E. Higgins, G. Tellez, and B. M. Hargis. 2007. Effect of a *Lactobacillus* spp.-based probiotic and dietary lactose prebiotic on turkey poult performance with or without *Salmonella* Enteritidis challenge. *J. Appl. Poult. Res.* 16:361–364.
- World Health Organization. 2006. Drug resistant *Salmonella*. Accessed Apr. 21, 2010. <http://www.who.int/mediacentre/factsheets/fs139/en/>.
- Yan, F., R. Angel, and C. M. Ashwell. 2007. Characterization of the chicken small intestine type IIB sodium phosphate cotransporter. *Poult. Sci.* 86:67–76.
- Zhang, S., H. S. Lillehoj, C. H. Kim, C. L. Keeler, U. Babu, and M. Z. Zhang. 2008. Transcriptional response of chicken macrophages to *Salmonella enterica* serovar Enteritidis infection. *Dev. Biol. (Basel)* 132:141–151.
- Zhou, H., and S. J. Lamont. 2007. Global gene expression profile after *Salmonella enterica* Serovar *enteritidis* challenge in two F8 advanced intercross chicken lines. *Cytogenet. Genome Res.* 117:131–138.