Effect of dietary nonphytate phosphorus content on ileal lymphocyte subpopulations and cytokine expression in the cecal tonsils and spleen of laying hens that were or were not orally inoculated with Salmonella Typhimurium

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OBJECTIVE
To evaluate the effects of dietary nonphytate phosphorus (NPP) content on ileal lymphocyte subpopulations and cytokine expression in the cecal tonsils and spleen of hens that were or were not inoculated with Salmonella Typhimurium.

ANIMALS
64 Salmonella-free hens.

PROCEDURES
Hens were fed a diet with 0.22% (control; n = 32) or 0.42% (high-P; 32) NPP for 6 weeks and then orally inoculated with S Typhimurium (5 X 10^7 CFUs) or PBSS. Tissues were obtained from 8 S Typhimurium–inoculated and 8 PBSS–inoculated hens from each group at 2 and 7 days postinoculation (DPI). Percentages of ileal CD4+ and CD8+ lymphocytes were determined by flow cytometry. Cytokine mRNA expression was determined by quantitative real-time PCR assays.

RESULTS
For S Typhimurium–inoculated hens, plasma parathyroid hormone concentration was significantly increased and 1,25-dihydroxyvitamin D3 concentration was decreased in hens fed the high-P diet, compared with values in hens fed the control diet. Salmonella Typhimurium inoculation caused an increase in the percentage of ileal CD8+ lymphocytes and the expression of interferon-γ, IL-1β, IL-6, IL-8, interferon-γ, IL-12, and IL-18 in the cecal tonsils and spleen and a decrease in the expression of IL-4 and IL-10 in the cecal tonsils. Hens fed the high-P diet had significantly increased splenic expression of interferon-γ at 2 DPI and IL-1β, IL-6, IL-12, and IL-18 at 7 DPI, compared with hens fed the control diet.

CONCLUSIONS AND CLINICAL RELEVANCE
Results suggested there was a T-helper 1 cytokine reaction in the cecal tonsils and spleen of S Typhimurium–inoculated hens, and dietary NPP content altered calcium regulation hormone concentrations and affected splenic cytokine expression. (Am J Vet Res 2015;76:710–718)

Phosphorus is an essential mineral that is involved in energy production, muscle and skeletal growth, and amino acid and carbohydrate metabolism in animals. Although the National Research Council’s recommended dietary NPP requirement for White Leghorns is 250 mg/hen/d (dependent on performance and bone strength), most commercial diets for laying hens are formulated with high concentrations of phosphorus (0.34% to 0.45% NPP; 450 mg/hen/d) to avoid phosphorus deficiency. Results of multiple studies indicate no significant differences in the performance and egg quality among hens fed diets with NPP contents ranging from 0.20% to 0.45%; however, hens fed a 0.45% NPP diet had significantly higher phosphorus excretion, compared with hens fed diets with lower NPP contents. Dietary NPP content is positively correlated with plasma IgM concentration in European whitefish, antibody titers against sheep blood cells in broiler chickens, and the cell-mediated immune response in pigs. The effect of high dietary NPP content on the immunity of laying hens is poorly understood.

ABBREVIATIONS
DPI  Days postinoculation
HBSS  Hanks balanced salt solution
IFN  Interferon
IL  Interleukin
NPP  Nonphytate phosphorus
PTH  Parathyroid hormone
Th  T helper
Consumption of meat and eggs contaminated with bacteria, particularly *Salmonella enterica* serovar Typhimurium, is a major source of foodborne infections in humans. Therefore, control of *Salmonella* infection in chicken flocks is an important public health issue. In mammalian species, phosphorus status affects the immune response to *Salmonella* infection. The susceptibility of guinea pigs to experimental infection with virulent *S. Typhimurium* was inversely associated with dietary phosphorus concentration. Three days after experimental infection, the clearance rate of *S. Typhimurium* by the reticuloendothelial system for guinea pigs fed a diet with 1.0% phosphorus was significantly greater than that of guinea pigs fed a diet with 0.4% phosphorus. To our knowledge, information regarding the effect of dietary NPP content on the immune response in chickens infected with *Salmonella* spp is lacking.

In avian species, cytokines have an integral role in the immune response against *Salmonella* infection. Changes in cytokine expression following infection with *Salmonella* spp have been investigated in avian epithelial cells and monocytes in vitro and in newly hatched chickens in vivo. Interferon-γ, IL-12, and IL-18 are associated with a protective immune response, whereas IL-4 and IL-10 aid in the downregulation of the inflammatory response against *Salmonella* spp. The aim of the study reported here was to investigate the effect of high dietary concentrations of NPP on the intestinal lymphocyte subpopulations and the expression of various cytokines in the cecal tonsil and spleen of laying hens experimentally infected with *S. Typhimurium*.

**Materials and Methods**

**Animals**

Sixty-four 50-week-old Roman hens were selected from a *Salmonella*-free flock. Hens that were not laying eggs or laying eggs with defective shells were excluded from the study population. All hens were seronegative for antibodies against *Salmonella* spp as determined by a commercial ELISA that was performed in accordance with the manufacturer’s instructions. Cloacal swab specimens were obtained from each hen and cultured for *Salmonella* spp as described to ensure that the selected hens were *Salmonella*-free. The hens were transferred to adjacent individual cages in an environmentally controlled biosafety level 2 building and allowed to acclimate for 7 days. Hens had ad libitum access to food and water and were maintained in 16 hours of light and 8 hours of darkness each day. All study protocols were approved by and conducted under the guidelines of the Animal Health and Care Committee of Sichuan Agricultural University.

**Diets**

Hens were randomly allocated by means of a computerized random-number generator to receive a diet that contained either 0.22% (control diet; n = 32) or 0.42% (high-P diet; 32) NPP for 6 weeks (Appendix I). The diets were formulated to contain adequate concentrations of all nutrients as recommended by the National Research Council and did not contain any antimicrobial agents.

**Salmonella Typhimurium strain**

A spontaneous amikacin- and neomycin-resistant strain of *S. Typhimurium* SC0906 obtained from the Veterinary Laboratory of Sichuan Agricultural University was used in the study. *Salmonella Typhimurium* SC0906 is virulent in young chicks < 7 days old and is capable of persistent colonization of the gastrointestinal tract of older birds. The bacteria were maintained in glycerol stock solution at –70°C. Prior to the experimental inoculation of hens, the stock bacteria solution was thawed and inoculated into sterile tubes that contained 10 mL of sterile tryptone soy broth, and that *S. Typhimurium* preculture solution was incubated at 37°C for 18 hours. Subsequently, 5 mL of the *S. Typhimurium* preculture solution was transferred to 100 mL of tryptone soy broth and shaken and incubated at 37°C for 18 hours. To determine the concentration of viable *S. Typhimurium* in the culture solution, 1-mL aliquots were serially diluted with sterile PBSS (pH 7.2) to dilutions of 1:10² to 1:10⁹, streaked on a culture plate containing xylose lysine deoxycholate agar, and incubated at 37°C for 24 hours. Following incubation, black-colored bacterial colonies were counted, and the *S. Typhimurium* culture solution was diluted with PBSS as necessary to achieve a concentration of 5 × 10⁷ CFUs of *S. Typhimurium/mL.*

**Inoculation procedure**

After the assigned diet had been fed for 6 weeks, 16 hens in the control and high-P diet groups were inoculated with 5 × 10⁷ CFUs of *S. Typhimurium* (1 mL of the culture solution), and the remaining 16 hens in each group were inoculated with 1 mL of PBSS (pH, 7.2). For both inoculations, a flexible tube was orally passed into the crop and a syringe was used to instill the assigned inoculant directly into the crop. Within each group, the *S. Typhimurium*-inoculated hens were isolated in a room separate from the PBSS-inoculated hens; however, the light-and-dark schedule, temperature, and other rearing conditions were held constant for all hens.

**Sample collection**

Within both the control and high-P groups, 8 *S. Typhimurium*-inoculated and 8 PBSS-inoculated hens were euthanized by means of IV injection of sodium pentobarbital (20 mg/kg) at 2 and 7 DPI. From each hen, 5 mL of blood was obtained from the right wing for determination of plasma biochemical variables associated with phosphorus metabolism. A 5-cm portion of the ileum approximately 5 cm proximal to the ileocecal junction was obtained aseptically for isolation of ileal lymphocytes. The cecal tonsils and spleen were obtained
and frozen in liquid nitrogen, then stored at -80°C until analyzed for cytokine expression by means of RNA extraction and measurement.

**Plasma biochemical variables**

Plasma calcium and phosphorus concentrations were determined by the use of commercial assay kits6 and an automated biochemical analyzer.7 Plasma PTH concentration was determined by use of a radioimmunoassay.8 Plasma 1,25-dihydroxyvitamin D$_3$ concentration was determined by use of a commercially available ELISA.9

**Ileal lymphocyte subpopulations**

For each ileum specimen, the mucosa was scraped and washed with ice-cold HBSS without calcium chloride and magnesium sulfate.1 The mucosa was then treated with 10mM dithiothreitol1 in calcium- and magnesium-free HBSS with 5% fetal bovine serum1 and incubated in 100µM EDTA in calcium- and magnesium-free HBSS with 5% fetal bovine serum at 37°C for 20 minutes with continuous swirling. The solution was then gently squeezed through a nylon mesh by use of a syringe plunger to prepare the cell suspension. The cell suspension was diluted with lymphocyte separation medium (density, 1.077 g/mL) and centrifuged at 200 X g for 20 minutes. The lymphocyte layer was collected and transferred to another centrifuge tube to which 2 mL of PBSS was added. The tube was centrifuged at 200 X g for 5 minutes, and the supernatant was discarded. The number of lymphocytes in the remaining pellet was calculated by use of a Neubauer counting chamber.4 The trypan blue dye exclusion method was used to determine cell viability, which was consistently > 95%. Viable cells were diluted with PBSS to achieve a concentration of 1.0 X 10$^6$ cells/mL, and 1 mL of the cell suspension was transferred to another centrifuge tube and centrifuged at 200 X g for 5 minutes. The supernatant was discarded. The cells were stained with 10 µL of mouse anti-chicken CD4 coupled to phyto-erythrin and mouse anti-chicken CD8α coupled to fluorescein isothiocyanate4 for 15 minutes at room temperature (22 ± 2°C). Then 2 mL of PBSS was added to the tube and centrifugal elutriation was performed once. The supernatant was discarded. The cells were resuspended in 0.5 mL of PBSS, and within 45 minutes, the numbers of CD4+ and CD8+ lymphocytes in the suspension were determined by flow cytometry.40

**Quantitative real-time PCR assay**

A qualitative real-time PCR assay6 was performed to extract the total RNA from each cecal tonsil and spleen specimen as described.21,22 The primers and annealing temperatures used for IL-1β, IL-6, IFN-γ, IL-4, IL-8, IL-10, IL-12, IL-18, and β-actin were summarized (Appendix 2). Amplification was conducted with denaturation for 15 minutes at 95°C, followed by 40 cycles of denaturation for 5 seconds at 95°C, and annealing and elongation for 30 seconds at specific temperatures for the various genes, and a final melting curve analysis. All reactions were run in duplicate, and the real-time PCR assay efficiency for each gene was calculated from the slope of the standard curve for that gene. The corresponding real-time PCR assay efficiency (E) of 1 cycle in the exponential phase was calculated as $E = 10^{-1/slope}$. The relative expression ratio of a target gene was calculated from $E$ and the cycle number of the crossing point (CP) deviation of the unknown sample versus a calibrator, and expressed in comparison to a reference gene.25

$$\text{Ratio} = \frac{(E_r) \Delta CP_{\text{target}}}{(E_r) \Delta CP_{\text{reference}}}$$

where $E_r$ is the real-time PCR assay efficiency of the target gene transcript, $E_r$ is the real-time PCR assay efficiency of the reference gene transcript, $\Delta CP_{\text{target}}$ is the CP deviation of the calibrator – sample of the target gene transcript, and $\Delta CP_{\text{reference}}$ is the CP deviation of the calibrator – sample of the reference gene transcript.

**Statistical analysis**

Descriptive statistics were calculated for each outcome of interest (plasma calcium, phosphorus, PTH, and 1,25-dihydroxyvitamin D$_3$ concentrations; percentage of CD4+ and CD8+ lymphocytes in the ileal mucosa; and IL-1β, IL-6, IFN-γ, IL-4, IL-8, IL-10, IL-12, IL-18, and β-actin expression in the cecal tonsils and spleen). The distributions of the data for each outcome were analyzed for normality by means of a univariate procedure as described.24 Some data were transformed to improve the normality of the distributions for parametric analysis. Data for IL-8, IFN-γ, and IL-12 expression in the cecal tonsils were logarithmically transformed, and the inverse square root transformation was applied to the data for IL-10 expression in the cecal tonsil and IL-8, IFN-γ, IL-18 expression in the spleen. The study had a randomized complete design, and each outcome of interest was analyzed with a mixed ANOVA as described.24 Main (fixed) effects included in each model included dietary NPP content, inoculant (PBSS or S Typhimurium), postinoculation time (2 or 7 DPI), and all possible 2-way interactions among the main effects. Random effects included the effect of each subject, and the interactions between subject and dietary NPP content and between subject and inoculant. Differences among means were tested by the least significant difference method. All analyses were performed with a commercially available statistical software program,26 and values of $P < 0.05$ were considered significant.

**Results**

**Plasma biochemical variables**

Plasma concentrations of calcium, phosphorus, PTH, and 1,25-dihydroxyvitamin D$_3$ for hens in each treatment group (DPI-inoculant-diet combination)
were summarized (Table 1). The plasma calcium, PTH, and 1,25-dihydroxyvitamin D₃ concentrations were significantly affected by the dietary NPP content, inoculant (PBSS or S. Typhimurium), and the interaction between dietary NPP content and inoculant, whereas the plasma phosphorus concentration was significantly affected only by the inoculant. For hens inoculated with S. Typhimurium, the plasma calcium and phosphorus concentrations were significantly decreased, compared with those for hens inoculated with PBSS, but did not differ significantly between hens at 2 and 7 DPI. Inoculation of hens with S. Typhimurium significantly increased plasma 1,25-dihydroxyvitamin D₃ concentration; however, the magnitude of that increase was greater for the hens fed the control diet, compared with that for hens fed the high-P diet. Inoculation with S. Typhimurium also increased the plasma PTH concentration of hens fed the high-P diet but did not significantly affect the plasma PTH concentration of hens fed the control diet.

### Intestinal lymphocyte subpopulations

The percentage of CD4⁺ lymphocytes in the ileal mucosa was significantly affected by the dietary NPP content (P < 0.001), inoculant (P = 0.004), number of DPI (P < 0.001), and the interaction between dietary NPP content and inoculant (P = 0.049). The percentage of ileal CD4⁺ lymphocytes was significantly greater in hens fed the high-P diet versus hens fed the control diet regardless of the inoculant (Figure 1). Following inoculation with S. Typhimurium, hens fed the high-P diet had a greater percentage of ileal CD4⁺ lymphocytes than did hens fed the control diet, and the percentage of CD4⁺ lymphocytes for hens fed the high-P diet at 2 DPI was significantly greater than that for hens fed the control diet at 7 DPI.

The percentage of CD8⁺ lymphocytes in the ileal mucosa was significantly affected by the dietary NPP content (P < 0.001) and the inoculant (P < 0.001). Hens fed the high-P diet had a significantly greater percentage of ileal CD8⁺ lymphocytes than did hens fed the control diet (Figure 1). Inoculation with S. Typhimurium also significantly increased the percentage of ileal CD8⁺ lymphocytes regardless of the diet fed.

### Cytokine expression in the cecal tonsils

The expression of mRNA for all genes evaluated was normalized to the expression of mRNA for β-actin (ie, relative expression). The relative expression of mRNA for the cytokines evaluated in the cecal tonsils...
was summarized (Table 2). The inoculant significantly (P < 0.001) affected the expression of all cytokines evaluated. Compared with hens inoculated with PBSS, hens inoculated with S. Typhimurium had significantly increased expression of IL-1β, IL-6, IFN-γ, and IL-18 and decreased expression of IL-10. Hens inoculated with S. Typhimurium had increased expression of IL-8 regardless of the diet fed, and the magnitude of that increase was significantly greater at 7 DPI than at 2 DPI. Similarly, hens inoculated with S. Typhimurium had significantly increased expression of IL-12, and the magnitude of that increase was significantly greater at 7 DPI than that at 2 DPI. Expression of IL-12 was also significantly affected by an interaction between dietary NPP content and inoculant. Expression of IL-4 was significantly decreased in hens fed the high-P diet and inoculated with S. Typhimurium, compared with that in hens fed the control diet and inoculated with PBSS, which indicated a significant interaction between dietary NPP content and inoculant.

### Cytokine expression in the spleen

Cytokine expression in the spleen varied from that in the cecal tonsils (Table 3). Expression of IL-12 was significantly affected by all main factors evaluated (ie, dietary NPP content, inoculant, number of DPI, and the interactions between dietary NPP content and number of DPI, between dietary NPP content and inoculant, and between inoculant and number of DPI), whereas expression of IL-4 was not significantly affected by any of the main effects. Expression of IL-1β in the spleen was significantly affected by dietary NPP content (P = 0.013) and inoculant (P < 0.001). Expression of IL-1β was greater in hens fed the high-P diet, compared with that in hens fed the control diet, and S. Typhimurium–inoculated hens had significantly increased IL-1β expression, compared with that of PBSS-inoculated hens. Expression of IL-6 and IL-18 was significantly affected by dietary NPP content, inoculant, and the interaction between dietary NPP content and inoculant. Splenic expression of both IL-6 and IL-18 was generally greater in hens fed the high-P diet, compared with that in hens fed the control diet, and was significantly greater in hens inoculated with S. Typhimurium, compared with that in hens inoculated with PBSS. Expression of IL-8 was significantly affected by inoculant (P < 0.001), number of DPI (P < 0.001), and the interaction between inoculant and number of DPI (P < 0.001). Splenic expression of IL-8 in hens inoculated with S. Typhimurium was generally greater than that in hens inoculated with PBSS, and the magnitude of that increase was greater in hens at 7 DPI than in hens at 2 DPI. Expression of IL-10 was significantly affected by inoculant (P = 0.005), number of DPI (P = 0.010), and the interactions between dietary NPP content and number of DPI (P = 0.006) and between inoculant and number of DPI (P < 0.001). Splenic expression of IL-10 was generally greater in hens inoculated with S. Typhimurium, compared with that in hens inoculated with PBSS, and the magnitude of that increase was greater in hens at 7 DPI that were fed the high-P diet, compared with that in hens at 2 DPI that were fed the control diet. Expression of IFN-γ was significantly affected by dietary NPP content (P = 0.011),

### Table 2—Mean ± SD values for relative expression of the mRNA of various cytokines in the cecal tonsils for the hens of Table 1.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Control diet PBSS</th>
<th>High-P diet S Typhimurium</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>4.62 ± 2.15a</td>
<td>3.73 ± 1.36a</td>
</tr>
<tr>
<td>IL-6</td>
<td>4.82 ± 0.66a</td>
<td>3.89 ± 0.90a</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>4.64 ± 1.76a</td>
<td>3.89 ± 0.90a</td>
</tr>
<tr>
<td>IL-12</td>
<td>4.62 ± 2.15a</td>
<td>3.73 ± 1.36a</td>
</tr>
<tr>
<td>IL-18</td>
<td>4.77 ± 2.73a</td>
<td>3.89 ± 0.90a</td>
</tr>
</tbody>
</table>

Table 2—Mean ± SD values for relative expression of the mRNA of various cytokines in the cecal tonsils for the hens of Table 1.

### Table 3—Mean ± SD values for relative expression of the mRNA of various cytokines in the spleen for the hens of Table 1.

<table>
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</table>

See Tables 1 and 2 for key.
inoculant \((P < 0.001)\), number of DPI \((P < 0.001)\), and the interaction between inoculant and number of DPI \((P < 0.001)\). Splenic expression of IFN-\(\gamma\) did not differ significantly among hens inoculated with PBSS regardless of diet or number of DPI. Inoculation of hens with \(S\) Typhimurium tended to cause a significant increase in IFN-\(\gamma\) expression, and the magnitude of that increase was greater at 7 DPI than at 2 DPI.

**Discussion**

Results of the present study suggested that there was a Th1–cytokine reaction in the cecal tonsils and spleen of \(S\) Typhimurium–inoculated hens, and dietary NPP content altered the plasma concentrations of calcium regulation hormones and affected splenic cytokine expression. Thus, feeding laying hens a diet with a high NPP content might improve the immune response of hens exposed to \(S\) Typhimurium.

Characterization of the metabolic effects associated with an immunologic challenge is important for the maintenance of nutrient homeostasis during an infection. The immune response to an infection generally results in the release of various cytokines from activated macrophages. In the present study, plasma calcium and phosphorus concentrations were significantly decreased in hens orally inoculated with \(S\) Typhimurium, compared with those in hens inoculated with PBSS. This finding was similar to results of another study in which the serum calcium and phosphorus concentrations of cows following intramammary infusion of lipopolysaccharide were significantly decreased, compared with those of cows following intramammary infusion of saline (0.9% NaCl) solution. Serum calcium and phosphorus concentrations are likewise decreased in dairy cows following IV infusion of *Escherichia coli* endotoxin. Additionally, in the present study, hens fed the high-P diet and inoculated with \(S\) Typhimurium had significantly greater plasma PTH and 1,25-dihydroxyvitamin D\(_3\) concentrations than did hens inoculated with PBSS, which suggested that the immune response of hens against \(S\) Typhimurium might activate osteolysis by osteoclasts, thereby increasing mobilization of calcium and phosphorus from bone. To our knowledge, studies conducted to investigate the effect of immune system activation on macromineral homeostasis are lacking. It is possible that the decrease in plasma calcium and phosphorus concentrations observed in the \(S\) Typhimurium–inoculated hens of the present study was caused by the immune response to the bacterium, which resulted in an increased demand for calcium and phosphorus to meet requirements for cellular energy metabolism and nucleic acid formation.

Cell-mediated immunity is critical for the protection of young chickens against \(S\) Typhimurium infection, but the cell-mediated immune response of mature laying hens against *Salmonella* infection remains to be elucidated. In the present study, laying hens orally inoculated with \(S\) Typhimurium had a significantly greater proportion of ileal CD8+ lymphocytes at 2 and 7 DPI than did hens inoculated with PBSS. This finding suggested that \(S\) Typhimurium inoculation caused a strong cell-mediated immune response in hens. A similar increase in the proportion of CD8+ lymphocytes in the cecum and spleen has been observed in young chickens following inoculation with \(S\) Typhimurium or *Salmonella Enteritidis*; however, the time required after inoculation for that increase to develop (4 to 5 DPI) was longer than that required (2 DPI) in the mature hens of the present study. This delay in the cell-mediated response of young chickens might simply be the result of an immature intestinal immune system. Regardless, results of the present study and those other studies indicated that the important role of CD8+ lymphocytes in the neutralization or killing of \(S\) Typhimurium in chickens is similar to that described in mammalian species.

In the present study, the percentage of CD4+ lymphocytes at 7 DPI was also significantly increased for \(S\) Typhimurium–inoculated hens, compared with that for PBSS-inoculated hens. A similar increase in the percentage of intestinal CD4+ lymphocytes was observed in naïve mice that were inoculated with virulent \(S\) Typhimurium. The increase in the proportion of CD4+ lymphocytes in the intestine might enhance the cytotoxic immune response that mediates the activation of CD8+ lymphocytes and macrophages in hens infected with \(S\) Typhimurium in a manner analogous to that described in mice.

Hens fed the high-P diet had an increase in the proportions of CD8+ and CD4+ lymphocytes in the ileal mucosa irrespective of whether they were inoculated with \(S\) Typhimurium. The addition of phytase to the diet of broilers increases phosphorus release, which in turn causes an increase in the proportion of CD4+ and CD8+ T cells in the blood. The percentage of circulating leukocytes was greater in guinea pigs fed a diet with a high phosphorus content, compared with that in guinea pigs fed a control diet. Results of multiple studies indicate that PTH stimulates various components of the immune system including peripheral blood lymphocytes and causes T lymphocyte proliferation. In the present study, the plasma calcium concentration for hens fed the high-P (0.42% NPP) diet was significantly lower than that for PBSS-inoculated hens. A similar increase in the percentage of intestinal CD4+ and CD8+ T cells has been observed in young chickens following inoculation with \(S\) Typhimurium in chickens is similar to that described in mice. In guinea pigs fed a diet with a high phosphorus content, compared with that in guinea pigs fed a control diet. Results of multiple studies indicate that PTH stimulates various components of the immune system including peripheral blood lymphocytes and causes T lymphocyte proliferation. In the present study, the plasma calcium concentration for hens fed the high-P (0.42% NPP) diet was significantly lower than that for PBSS-inoculated hens. A similar increase in the percentage of intestinal CD4+ and CD8+ T cells has been observed in young chickens following inoculation with \(S\) Typhimurium in chickens is similar to that described in mice.
increase in the mRNA expression of IL-1β, IL-6, and IL-8 in the cecal tonsils and spleen at 2 and 7 DPI, and were similar to findings of other studies\cite{16,17} that involved newly hatched chicks. Activation of macrophages and T lymphocytes by IL-6 may prime the acquired immune system,\cite{49} and IL-8 may act as a chemokine to recruit heterophils in chickens.\cite{57} Hens that were inoculated with S Typhimurium had greater recruitment of immune cells in the intestine than did hens inoculated with PBSS, and had greater expression of IL-6 and IL-8 in the cecal tonsils at 7 DPI than at 2 DPI, which likely was a reflection of the late expression pattern for those cytokines.\cite{57} Interferon-γ has a critical role in the elimination of intercellular pathogens such as Salmonella spp\cite{41,42} because it is a necessary Th1 cytokine for macrophage activation.\cite{45} Expression of IFN-γ was increased in the cecal tonsils and spleen at 2 and 7 DPI in the S Typhimurium–inoculated hens of the present study, in the cecal tonsils of 1-day-old chicks that were inoculated with 10^7 CFUs of S Enteritidis,\cite{58} and in the spleen of 1-week-old chicks that were inoculated with 10^6 CFUs of S Typhimurium.\cite{59} Interleukin-12 and IL-18 induce production of IFN-γ,\cite{60,61} whereas IL-4 antagonizes IFN-γ function and suppresses the inflammatory immune response.\cite{62,63} IL-10 inhibits expression of major histocompatibility complex class 2 and costimulatory molecules.\cite{64} In the present study, inoculation of hens with S Typhimurium caused an increase in the expression of IL-12 and IL-18 in the cecal tonsils and had no apparent effect on IL-4 and IL-10 expression, which indicated an important adaptation by the immune system to increase antigen presentation capability and mount a proinflammatory response.\cite{65} Inoculation of young chicks with various S enterica serovars also increased expression of IL-12 and IL-18 in the cecal tonsils,\cite{66,67}; however, inoculation of young chicks with S Typhimurium did not affect IL-10 expression in the cecal tonsils at 1 and 5 DPI.\cite{66,67} The prominent increase in IFN-γ expression and concurrent increase in the expression of IL-12 and IL-18 detected in the S Typhimurium–inoculated hens of the present study and young chicks of other studies\cite{57,61} indicate the importance of the Th1 immune response against Salmonella infection.

For the PBSS-inoculated hens of the present study, dietary NPP content did not affect the expression of any cytokine in the cecal tonsils except IL-4, which was significantly decreased in hens fed the high-P diet, compared with that in hens fed the control diet. Conversely, for the S Typhimurium–inoculated hens, splenic expression of IFN-γ at 2 DPI and IL-12 and IL-18 at 7 DPI was increased in hens fed the high-P diet, compared with that in hens fed the control diet. These results suggested that the Th1 immune response of laying hens against Salmonella infection was enhanced by an increase in dietary NPP intake. 1,25-dihydroxyvitamin D_3 suppresses IL-2 production\cite{68} and inhibits the development of Th1 cells that produce IFN-γ.\cite{69,70} The lower plasma 1,25-dihydroxyvitamin D_3 concentrations observed in hens fed the high-P diet, compared with those in hens fed the control diet, likely contributed to the increase in the splenic expression of Th1 cytokines in hens that were inoculated with S Typhimurium. In the present study, IL-4 expression in the cecal tonsils of hens fed the high-P diet was significantly decreased, compared with that of hens fed the control diet. Parathyroid hormone interacts with IL-4 to inhibit immunoglobulin production.\cite{71} Consequently, high dietary NPP content might decrease IL-4 expression, which in turn could reduce the inhibition of immunoglobulin production by PTH and enhance the adaptive immune response.

In the present study, inoculation of laying hens with S Typhimurium induced a cell-mediated immune response that was characterized by an increase in the expression of various Th1 cytokines in the cecal tonsils and spleen. That immune response was enhanced in hens fed a diet with a high (0.42%) NPP content versus hens fed a diet with a low (0.22%) NPP content.

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**Footnotes**


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h. DRG Instruments GmbH, Marburg, Germany.

i. TaKaRa, Dalian, China.

j. Zhejiang Tianhang Biological Technology Co Ltd, Hanzhou, China.

k. Haussner Scientific, Blue Bell, Pa.

l. Pharmingen, BD, Franklin Lakes, NJ.

m. FACSCalibur, BD, Franklin Lakes, NJ.


o. PROC MIXED, SAS, version 9.3, SAS Institute Inc, Cary, NC.

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26. Weibel DM, Finck BN, Baker DH, et al. Time course of increased plasma cytokines, cortisol, and urea nitrogen in pigs follow-
Appendix 1

Composition and nutrient concentrations of control (NPP content, 0.22%) and high-P (NPP content, 0.42%) diets fed to laying hens.

<table>
<thead>
<tr>
<th>Items</th>
<th>Control diet</th>
<th>High-P diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn</td>
<td>59.48</td>
<td>59.48</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>27.59</td>
<td>27.59</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>1.50</td>
<td>1.50</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>0.09</td>
<td>0.09</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.40</td>
<td>0.40</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Premix*</td>
<td>0.53</td>
<td>0.53</td>
</tr>
<tr>
<td>Mono-dicalcium phosphate</td>
<td>0.47</td>
<td>1.42</td>
</tr>
<tr>
<td>Limestone</td>
<td>8.28</td>
<td>7.90</td>
</tr>
<tr>
<td>Bentonite</td>
<td>1.56</td>
<td>0.99</td>
</tr>
<tr>
<td>Metabolizable energy (kcal/kg)</td>
<td>2,688</td>
<td>2,688</td>
</tr>
<tr>
<td>Crude protein</td>
<td>16.50</td>
<td>16.50</td>
</tr>
<tr>
<td>Calcium</td>
<td>3.48</td>
<td>3.48</td>
</tr>
<tr>
<td>Methionine and cystine</td>
<td>0.61</td>
<td>0.61</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.34</td>
<td>0.34</td>
</tr>
<tr>
<td>NPP†</td>
<td>0.22</td>
<td>0.42</td>
</tr>
</tbody>
</table>

Values represent the percentage of the diet on an air-dried basis unless otherwise indicated.
*Supplied the following per kilogram of complete feed: iron, 60 mg; copper, 8 mg; manganese, 100 mg; zinc, 100 mg; selenium, 0.3 mg; iodine, 0.35 mg; vitamin A (retinyl palmitate), 8,000 U; cholecalciferol, 1,600 U; vitamin E (d-tocopheryl acetate), 5 U; thiamine, 0.8 mg; riboflavin, 2.5 mg; pyridoxine, 1.5 mg; vitamin B12, 0.004 mg; pantethenic acid, 2.2 mg; folic acid, 0.25 mg; nicotinic acid, 20 mg; and biotin, 0.1 mg.
†Determined by triplicate analyses.

Appendix 2

Primers used for quantitative real-time PCR assays to determine cytokine mRNA expression in the cecal tonsils and spleen of laying hens.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers sequence (5′–3′)</th>
<th>Annealing temperature (°C)</th>
<th>Amplification size (bp)</th>
<th>GenBank accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>F: TGGGCTATGAAGGCGTACCA</td>
<td>60</td>
<td>244</td>
<td>Y15006</td>
</tr>
<tr>
<td></td>
<td>R: TCAGGTTTGGTTGTGATG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>F: CAAAGGTGACGGAGGACG</td>
<td>58</td>
<td>254</td>
<td>AJ095440</td>
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<tr>
<td></td>
<td>R: TGGCCAGGGAGGATTCT</td>
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</tr>
<tr>
<td>IL-8</td>
<td>F: GCCTGCTAGGGGAAATGAAG</td>
<td>60</td>
<td>136</td>
<td>AJ009800</td>
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<tr>
<td></td>
<td>R: GGAATTACCAGTTGCTG</td>
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<tr>
<td>IFN-γ</td>
<td>F: AGCTGACGTTGACATATT</td>
<td>57</td>
<td>259</td>
<td>Y07922</td>
</tr>
<tr>
<td></td>
<td>R: GGCCTTGGGCTTGATTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-4</td>
<td>F: ACCAGGGGCAATCCAGAAG</td>
<td>61</td>
<td>258</td>
<td>A621735</td>
</tr>
<tr>
<td></td>
<td>R: CAGTGGCAGGGAAGGACT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>F: CGGGAGTCTAGGGTGAAG</td>
<td>60</td>
<td>272</td>
<td>A621614</td>
</tr>
<tr>
<td></td>
<td>R: TGAAGAGGCGGTGACG</td>
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<tr>
<td>IL-12</td>
<td>F: AGACTCCAAATGGGACATGA</td>
<td>55</td>
<td>274</td>
<td>NM_213571</td>
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<tr>
<td></td>
<td>R: CTCCTCAGCCAAAGGACAGT</td>
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<tr>
<td>IL-18</td>
<td>F: GGAATGGCTAGCTTTTGG</td>
<td>54</td>
<td>264</td>
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</tr>
<tr>
<td></td>
<td>R: ATTTCCCCATGCCTTTTCTCA</td>
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<tr>
<td>β-actin</td>
<td>F: GAGAAATTTGCGTGCATCA</td>
<td>60</td>
<td>152</td>
<td>L08165</td>
</tr>
<tr>
<td></td>
<td>R: CCTGGACCTCTCTGGAACCA</td>
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</tbody>
</table>