

Effects of Nonimmunized Egg Yolk Powder–Supplemented Feed on *Salmonella* Enteritidis Prevention and Elimination in Broilers

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SUMMARY. Chicken consumption is a newly identified risk factor in *Salmonella enterica* serovar Enteritidis (SE) infection in humans. SE is widely distributed in commercial chicken flocks and high levels of cecal carriage and shedding may lead to broiler meat contamination. In the present study, the preventive and eliminative effect of nonimmunized freeze-dried egg yolk powder (EYP) on SE in broilers was investigated. In the prevention trial, reduced SE counts were observed in liver ($P \leq 0.05$), cecal contents, and fecal shedding ($P \leq 0.05$) in birds fed 10% or 5% EYP. Histological examination of cecal wall and cecal tonsils at 23 days postinfection indicated a lesser degree of intestinal pathology. In the elimination trial, a significantly lower ($P \leq 0.05$) number of SE reached the liver and spleen, and a reduction in cecal carriage and fecal shedding was observed. The histological changes in the cecal mucosa and cecal tonsils reflected an apparent inflammation and mucosal repair and also suggested that the infection had not completely resolved, confirming SE bacterial isolations in the cecal tissue. The present study indicates that supplementing the diets of broilers with 5% nonimmunized EYP, at the early stages of the growing period, reduces preharvest *Salmonella* load with a minimal degree of intestinal pathology.

RESUMEN. Efecto del alimento suplementado con yema de huevo en polvo no inmunizada en la prevención y eliminación de *Salmonella* Enteritidis en pollos de engorde.

El consumo de pollo es un factor de riesgo recientemente identificado en las infecciones con *Salmonella enterica* serovar Enteritidis en humanos. La *Salmonella enterica* serovar Enteritidis se encuentra ampliamente distribuida en las parvadas comerciales y altos niveles de contaminación cecal y de diseminación puede conllevar a la contaminación de la carne de pollo. En el presente estudio se investigó el efecto preventivo y eliminativo de la yema de huevo en polvo no inmunizada, sobre la *Salmonella enterica* serovar Enteritidis en pollos de engorde. En el experimento relacionado con la prevención, las aves alimentadas con 10% o 5% de yema de huevo en polvo mostraron una disminución en el recuento de *Salmonella enterica* serovar Enteritidis en el hígado, contenido cecal y diseminación en heces ($P \leq 0.05$). La evaluación histopatológica de las paredes del ciego y de las tonsilas cecales 23 días posteriores a la infección, indicó un menor grado de patologías intestinales. En el experimento relacionado con la eliminación, se observó una reducción de la contaminación cecal y de la diseminación fecal, mientras un número significativamente menor de *Salmonella enterica* serovar Enteritidis ($P \leq 0.05$) alcanzó el hígado y el bazo. Los cambios histológicos en la mucosa del ciego y las tonsilas cecales reflejaron una inflamación aparente y evidencia de reparación de la mucosa, así mismo sugirieron que la infección no se resolvió por completo, lo cual sirvió de confirmación para los aislamientos de *Salmonella enterica* serovar Enteritidis obtenidos en tejidos cecales. El presente estudio indica que la suplementación del alimento para pollos de engorde en etapas tempranas con 5% de yema de huevo en polvo no inmunizada reduce la carga de *Salmonella enterica* serovar Enteritidis y disminuye el grado de patologías intestinales.

Key words: *Salmonella* Enteritidis, nonimmunized egg yolk powder, broilers, histopathology, inflammation

Abbreviations: BGA = brilliant green agar; BHI = brain heart infusion; CFU = colony-forming units; EYP = egg yolk powder; GALT = gut-associated lymphoid tissue; H & E = hematoxylin and eosin; p.i. = postinfection; SE = *Salmonella enterica* serovar Enteritidis; TBG = tetrathionate brilliant green

A total of 1.4 million cases of nontyphoidal *Salmonella* Enteritidis cases are reported in the United States annually (18). Reports of sporadic cases of salmonellosis in the United States have revealed that consumption of contaminated eggs is a major risk factor (23). Recently, consumption of chicken meat has also been identified as a risk factor (12). *Salmonella enterica* serovar Enteritidis (SE) phage type 4 has been implicated in these outbreaks. In Canada, salmonellosis accounts for the second highest cases of enteric illnesses, with an incidence rate of 22.6 cases per 100,000 (16). Contaminated poultry and other meat items were also identified as vehicles for SE transmission to humans.

In the past, antimicrobials have been used to tremendously reduce bacterial infection and improve production performance efficiencies, but antimicrobial resistance has become an issue for the poultry industry. The emergence of various *Salmonella* strains with multi-resistant antibiograms resulted in a looming prohibition of antimicrobials in poultry. Currently, there is no single effective

alternative to the use of antimicrobials for *Salmonella* reduction in poultry flocks. However, information on the pathogenesis and virulence attributes of *Salmonella* tremendously increased in the last two decades. Several *Salmonella* reduction schemes have been considered, including the use of competitive exclusion products, prebiotics, acidifiers, live and inactivated vaccines, and immunomodulators. These schemes target one or more different points of control of the *Salmonella* infection cycle: Probiotics aim to reduce the population of *Enterobacteriaceae* in the gut (19,28); oligosaccharide prebiotics increase the number of beneficial bacteria in the gut, act as antiadhesives by binding to bacterial receptors, and modulate mucosal immunity (4,5,26,27); acidifiers reduce the gut pH and inhibit growth of *Salmonella* and other coliforms (24,29); live vaccines are used to improve the development of specific immunity to *Salmonella* (1,21,22); and immunomodulators target the innate immune system for improved heterophil and macrophage functions, e.g., *Salmonella* lymphokines (14,15), β -glucan (17), and CpG DNA (31). However, none of these has consistently reduced the levels of *Salmonella* and the combined use of one or more of the

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Table 1a. Pellet-crumble ingredient composition for broiler starter.

Ingredient	Regular starter	5% EYP starter	10% EYP starter
Nonimmunized egg yolk	0.000	5.000	10.000
Corn	58.637	60.573	59.603
Soybean meal (47% protein)	29.750	26.540	22.250
Corn gluten meal (60)	5.000	3.490	3.240
Animal and vegetable fat	2.510	0.000	0.000
Bicalcium phosphate	1.620	1.660	1.720
Calcium carbonate	1.290	1.610	2.120
Salt	0.470	0.480	0.480
DL-Methionine	0.189	0.147	0.097
Vitamin and mineral premix	0.500	0.500	0.500
L-Lysine	0.034	0.000	0.000
Total	100.000	100.000	100.000

above alternatives are oftentimes implemented in commercial flocks for improved prevention and elimination of the organism.

Recently, our group described the potential use of food proteins for the prevention and elimination of bacteria; nonimmunized egg yolk powder (EYP) was one of these (10). Hens that have been hyperimmunized with different pathogens or vaccines produce high levels of specific immunoglobulins in the yolk. The chicken egg can serve as an effective and practical vehicle for immunoglobulin production in oral immunotherapy. Other than functioning as a carrier, the egg yolk proteins have been useful for the prevention and elimination of enteric pathogens such as *Campylobacter jejuni*, SE, *Salmonella* Typhimurium, and *Escherichia coli* O157:H7 in layers (11). It has been reported that granules and high-density lipoprotein fractions have antiadhesive activity against these pathogens (9).

This paper describes *in vivo* experiments using broiler chickens with the following working objectives: 1) to determine the efficiency of EYP against SE infection in broilers and 2) to assess the histopathology of SE in EYP-fed birds. In two trials, we sought to determine the bird's response to SE if EYP is fed during the early stages of the growing period (prevention trial) prior to infection, or if birds are fed when they are already challenged (elimination).

Table 1b. Calculated composition of nutrients for starter rations.

Ingredient	Regular starter	5% EYP starter	10% EYP starter
Nonphytate phosphorus %	0.42	0.42	0.42
Calcium %	0.90	1.01	1.20
Crude protein %	22.00	22.00	22.00
Lysine %	1.10	1.10	1.10
Methionine %	0.53	0.52	0.50
Methionine + cysteine %	0.85	0.85	0.85
Arginine %	1.28	1.27	1.25
Threonine %	0.72	0.77	0.81
Tryptophan %	0.20	0.21	0.22
Isoleucine %	0.92	0.97	1.01
Valine %	1.03	1.08	1.14
Phenylalanine + tyrosine %	2.00	1.88	1.78
Metabolizable energy (kcal/kg)	3100.00	3100.00	3234.00
Na %	0.20	0.20	0.20
K %	0.78	0.72	0.64
Cl %	0.33	0.32	0.32
Crude fiber	2.55	2.44	2.25
Ash %	6.38	6.58	6.86
Linoleic acid %	1.82	1.57	1.63
Fat %	5.15	4.91	7.08

Table 2a. Broiler grower crumble and finisher formulations.

Ingredient (kg)	Regular grower	Regular finisher	5% EYP finisher
Nonimmunized EYP	0.000	0.000	5.000
Corn	63.071	68.608	71.413
Soybean meal (47% protein)	25.160	21.220	17.450
Corn gluten meal (60)	3.960	3.500	2.380
Animal and vegetable fat	3.730	2.870	0.000
Bicalcium phosphate	1.550	1.470	1.510
Calcium carbonate	1.360	1.160	1.160
Salt	0.470	0.470	0.480
DL-Methionine	0.124	0.088	0.057
Vitamin and mineral premix	0.500	0.500	0.500
L-Lysine	0.075	0.057	0.036
L-Threonine	0.000	0.058	0.014
Total	100.000	100.000	100.000

MATERIALS AND METHODS

Animals. Two hundred fifty-two 1-day-old chicks (Ross × Ross) vaccinated with Marek's disease virus and infectious bronchitis were obtained from Maple Leaf Foods Hatchery (New Hamburg, Ontario, Canada) and were screened for *Salmonella* spp. by cloacal swabbing prior to placement. Animals were housed at the Animal Isolation Unit, University of Guelph, Ontario, Canada. The animal experiments were conducted in accordance to the animal care guidelines and with the approval of the Animal Care Committee, University of Guelph.

Experimental diets. To prepare the nonimmunized EYP, eggs were obtained from the university research station; their exterior shell surfaces were disinfected, they were cracked, and the egg yolks were aseptically separated from albumen. Pooled egg yolks were freeze-dried and crushed into a fine powder. All egg yolk samples were tested for antibodies to SE by the enzyme-linked immunosorbent assay using formalin-killed whole SE cells as coating antigens. Samples were negative for the aforementioned antibodies. The SE whole cells for coating antigen were prepared as follows. Bacterial stock previously stored at -80 C in 20% glycerol was thawed and a loopful was plated on brilliant green agar (BGA; BD Diagnostic Systems, San Jose, CA). After an

Table 2b. Calculated composition of nutrients for broiler grower crumble and finisher formulations.

Ingredient (kg)	Regular grower	Regular finisher	5% EYP finisher
Nonphytate phosphorus %	0.40	0.38	0.38
Calcium %	0.90	0.80	0.80
Crude protein %	19.60	18.00	18.00
Lysine %	1.00	0.88	0.88
Methionine %	0.43	0.38	0.38
Methionine + cysteine %	0.72	0.64	0.64
Arginine %	1.15	1.08	1.06
Threonine %	0.64	0.64	0.64
Tryptophan %	0.18	0.16	0.17
Isoleucine %	0.81	0.73	0.78
Leucine %	1.97	1.83	1.79
Valine %	0.92	0.84	0.89
Phenylalanine + tyrosine %	1.77	1.61	1.49
Metabolizable energy (kcal/kg)	3200.00	3200.00	3307.00
Na %	0.20	0.20	0.20
K %	0.70	0.64	0.57
Cl %	0.34	0.33	0.33
Crude fiber %	2.45	2.41	2.31
Ash %	6.20	5.78	5.64
Linoleic acid %	2.08	2.05	1.76
Fat %	6.45	5.76	5.21

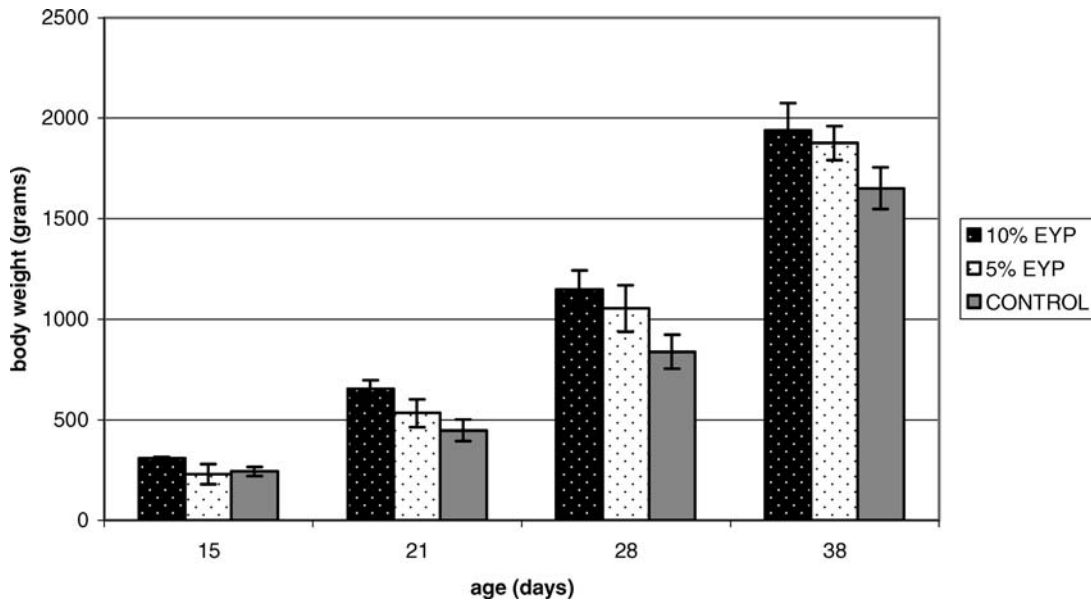


Fig. 1. Average body weight comparing three different diets on prevention trial. Vertical bars represent the standard error. $n = 3-4$. Diet effect: $P = 0.6040$, age effect: $P = 0.0001$, diet and age interaction: $P = 0.0962$.

overnight incubation at 37 C, a colony was inoculated in 500 ml of brain–heart infusion agar (BD Diagnostic Systems) and incubated at 37 C for 18 hr with shaking. The broth culture was centrifuged at $2500 \times g$ at 4 C for 5 min. The supernatant was discarded and the pellet was washed three times with ice-cold phosphate-buffered saline. The pellet was inactivated with 3.8% buffered formalin overnight at room temperature. After inactivation, the pellet was harvested by centrifugation and washed three times with ice-cold phosphate buffered saline to remove the formalin. The final pellet was frozen overnight at -80 C and freeze-dried for 24 hr. The nonimmunized EYP was mixed with the regular broiler starter (for prevention trials) or grower (for elimination trials) pellet-crumble without any antibiotics or anticoccidial drug (Tables 1, 2). All feeds used in this study were prepared at the Arkell Research Station, University of Guelph, Ontario, Canada.

Bacterial culture. *Salmonella enterica* serovar Enteritidis PT4 SA992212 resistant to novobiocin, a gift from Dr. Cornelius Poppe (Health Canada, Guelph, Ontario, Canada) and originally isolated from chickens was used in this study. The bacterial culture was retrieved from a bacterial stock and grown in BHI overnight at 37 C with shaking. The overnight culture was adjusted to 2×10^4 colony-forming units (CFU) per ml by calorimetric technique (Biomérieux Vitek, Inc., Hazelwood, MO) and confirmed by plating on BGA. The methods described in the U.S. Food and Drug Administration’s Bacteriological Analytical Manual (30) were used to confirm SE.

Animal experiments. The animal experiment was divided into two trials: prevention (Trial 1) and elimination (Trial 2).

Prevention (Trial 1). In this trial, the purpose of feeding EYP during the first 2 wk of the growing period was to determine if the feeding

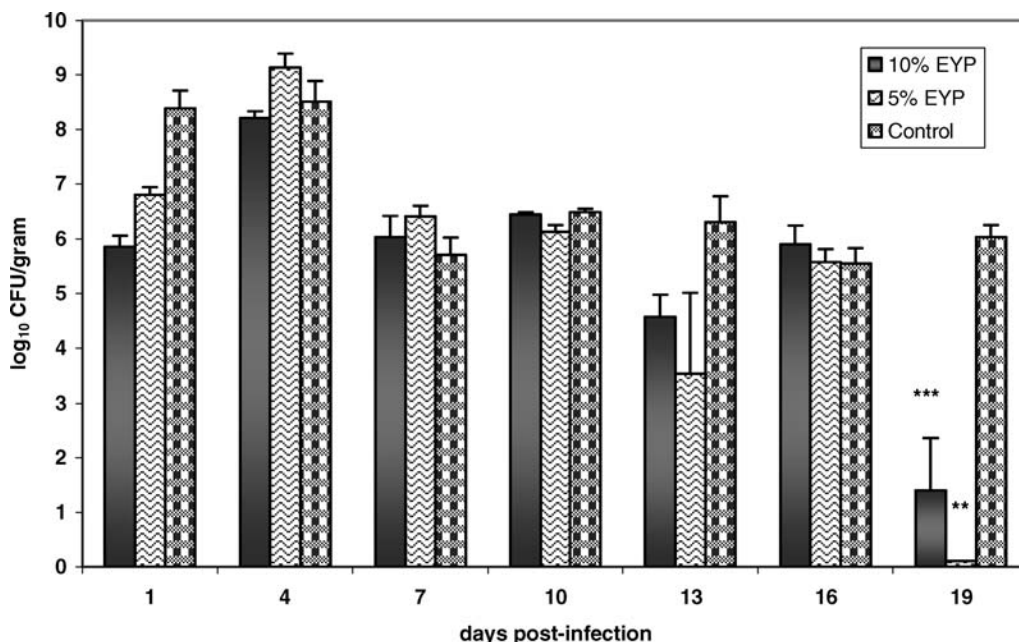


Fig. 2. Fecal shedding on prevention study. Mean \log_{10} CFU/g \pm SEM. ($n = 6$). *Indicates significant difference with the control ($P \leq 0.05$, Graphpad Instat®). **Indicates very significant difference ($P \leq 0.005$).

Table 3. Prevention trial; bacterial enumeration in organs.

Organs	Days postinfection			
	1	7	14	23
Liver				
10% EYP	5.85 ± 0.29 (3/3)	2.67 ± 0.86 (2/3)*	3.31 ± 0.14 (4/4)*	1.88 ± 0.79 (2/5)***
5% EYP	4.94 ± 0.09 (3/3)	3.38 ± 0.36 (1/3)***	1.42 ± 0.87 (1/3)***	0.70 ± 0.42 (1/3)***
Control	5.52 ± 0.17 (3/3)	4.89 ± 0.28 (3/3)	5.76 ± 0.29 (2/3)	6.51 ± 0.51 (3/3)
Cecal contents				
10% EYP	6.68 ± 0.57 (3/3)	4.79 ± 0.97 (3/3)	3.34 ± 1.06 (4/4)	3.91 ± 0.74 (3/5)
5% EYP	6.37 ± 0.57 (3/3)	4.93 ± 0.02 (3/3)	4.22 ± 0.12 (3/3)	3.56 ± 0.89 (2/3)
Control	7.50 ± 0.47 (3/3)	5.85 ± 1.04 (3/3)	4.99 ± 1.11 (2/3)	6.34 ± 1.98 (2/3)

^AMean log₁₀ CFU/g ± standard error of the mean (SEM).

^B() indicates proportion of positives.

*Indicates significant difference with the control ($P \leq 0.05$, Graphpad Instat®). ***Indicates highly significant difference with the control ($P \leq 0.0005$). All data are triplicate analyses.

could confer protection to SE infection in the growing birds until they were marketed. Three groups of 20 chicks were placed in wire cages with unlimited access to feed and water. Treatment diets were fed for 2 wk. Prior to inoculation, feed and water were withdrawn overnight. After this period, birds were then orally inoculated with 2×10^4 CFU/ml of SE. SE fecal shedding was monitored by fecal collection and culture every 2 days starting on the first day up to 21 days postinfection (p.i.). Postmortem was conducted in three to five birds per group prior to infection, and at days 1, 7, 14, and 23 days p.i. Various organs were collected for SE enumeration. Liver, spleen, bursa of Fabricius, cecal tonsils and ceca (cross-section) were selected for histopathological examination. The trial was performed twice.

Elimination (Trial 2). In this experiment, the birds were challenged when they reached 18 days of age, simulating field situations in growing broiler flocks infected halfway to harvest. A total of 22 chicks per group were placed in wire cages. Birds were allowed to have access to unlimited feed and water without any antibiotics or anticoccidial drugs. Birds were orally infected with 2×10^4 CFU/ml of SE. The level of infection was confirmed 24 hr after inoculation by organ culture and fecal shedding. Birds were subsequently fed with the treatment diet up to 17 days p.i., when a significant reduction in SE shedding was evident. Postmortem and sample collection was conducted as in Trial 1. The trial was performed twice.

Bacterial count. Overnight fecal droppings were collected in sterilized aluminum foil (12 × 12 inches) randomly placed in three

different locations in trays underneath the wire flooring. Twenty-five grams of feces from each cage was mixed with nine parts of tetrathionate brilliant green (TBG; BD Diagnostic Systems) broth supplemented with 0.02 µg/ml novobiocin (BD Diagnostic Systems) and thoroughly mixed (Osterizer®; Oster, Cincinnati, OH) for 5 min. Samples were incubated overnight at 41 C. Cultures were serially diluted 1:10 from 10^2 to 10^7 in 1% buffered peptone water (BD Diagnostic Systems) and plated in duplicates on BGA with 20 µg of novobiocin. Plates were incubated aerobically at 37 C overnight. For bacterial enumeration in organs and cecal contents, preweighed 10-ml culture tubes (Fisher Scientific, Neapan, Ontario) were used to collect samples during necropsy; samples were stored on ice, then nine parts TBG with novobiocin were added, and the mixture was homogenized for 1 min using Polytron® homogenizer (Brinkman Instruments, Westbury, NY). Succeeding steps were similar to the procedure described above, but Salmonella Shigella Agar (BD Diagnostic Systems) was used instead of BGA for plating.

Histopathology. At 23 days p.i., sections of the liver, spleen, bursa of Fabricius, cecal tonsils, and ceca (cross-section) were fixed in 10% buffered formalin for 24 hr, sectioned at 4-µm thickness, and embedded in paraffin. Slides were stained using routine hematoxylin and eosin (H & E) stain and Giemsa stain, then examined under light microscopy (Nikon® digital camera DXM 1200F microscope, equipped with Nikon Eclipse® E8000 camera, Melville, New York). A scoring system for the cecal tonsils was applied. The quantification of intraepithelial lymphocytes was based on the protocol described by Sheela *et al.* (25). Typical enteric *Salmonella* paratyphoid lesions were evaluated and were judged on the basis of inflammation, hyperplasia of mucous gland cells, intraepithelial mononuclear cells in the villi flanking the cecal tonsils, and population of lamina propria cells (7). The presence of granuloma was also recorded (6). In each of the three to five birds necropsied, five different intestinal villi flanking the cecal tonsils were selected at random and the following parameters were evaluated.

Inflammation (parameter a) was rated using the following scale: 1) mild, focal areas of hemorrhages and edema; 2) moderate, focal to multifocal areas of hemorrhages and edema; and 3) severe, multifocal coalescing areas of hemorrhages and edema. Mucous gland cells (parameter b) were rated as follows: 1) mild, focal areas of hyperplastic and hypertrophic mucous gland cells; 2) moderate, focal to multifocal areas with hyperplastic and hypertrophied mucous gland cells, mostly confined in hemorrhagic and edematous areas; and 3) severe, generalized hyperplasia and hypertrophy of mucous gland cells. Intraepithelial mononuclear cells (parameter c) were counted from five different microscopic fields at 40× magnification and ranked as follows: 1) mild, presence of less than 20 cells per microscopic field; 2) moderate, presence of 20 to 34 cells per microscopic field; and 3) increased, presence of 35 to 50 cells per microscopic field. Lamina propria cell population (parameter d) was evaluated as follows: 1) mild, lamina propria was

Table 4. Histopathological examination at 23 days p.i. in prevention trial.

Parameters	10% EYP	5% EYP	Control
Inflammation	Mild (0.50) ^{A*}	Mild (0.00)	Moderate (2.66)
Mucous gland cell population	Mild (1.44)	Mild (1.33)*	Moderate (2.00)
Intraepithelial mononuclear cells	Increased (3.00)*	Increased (3.00)*	Mild (1.00)
Lamina propria cell population	Increased (3.00)*	Increased (3.00)*	Moderate (1.66)
Other lesions:			
Necrosis of villous tips	Mild	Mild	Mild to moderate
Granuloma (cecal tonsils)	None	None	Yes

^AValues in parentheses are average histopathological scores.

*Indicates significant difference compared with the control ($P \leq 0.05$, Kruskal–Wallis for nonparametric analysis; Graphpad Instat®).

mildly infiltrated with mononuclear cells; 2) moderate, lamina propria was moderately infiltrated with mononuclear cells; and 3) increased, lamina propria was densely infiltrated with mononuclear cells consisting of mature lymphocytes, plasma cells, and macrophages. The slides stained with H & E were used in the evaluation of parameters a, b, and d. Giemsa-stained slides were used in the enumeration of intraepithelial mononuclear cells.

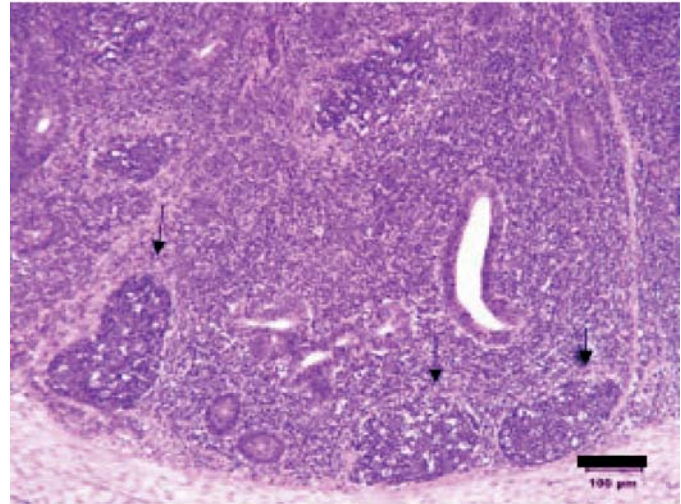
Statistical analysis. In the prevention trial, statistical differences between treatment groups were determined by one-way analysis of variance. The means derived from the quantification of bacteria from fecal samples and organs were analyzed on each sampling schedule and were further separated for significance with an all pair-wise multiple comparison applying the Tukey–Kramer test ($P \leq 0.05$, Graphpad Instat 3®, San Diego, California).

RESULTS AND DISCUSSION

Prevention trial. The birds were weighed before each necropsy to determine the diet effect on weight gain, because the feed nutrient compositions (Tables 1, 2) were slightly altered, particularly in 10% EYP-supplemented diet. The birds fed with 10% EYP exhibited the heaviest final body weight (average 1940 g), followed by the 5% EYP-fed birds (average 1877 g), and the control birds (average 1652 g; Fig. 1). The weight gain was influenced by age “effect” ($P = 0.0001$), and not as a result of diet and age interaction ($P = 0.0962$), or diet effect ($P = 0.6040$). The marginal effect of the diet may be because of the tendency of broilers to have slower weight gain during the first 2 wk of life, at the same period when the birds were exposed to the EYP-supplemented diet. A gradual reduction in fecal shedding was observed in both the EYP-fed and control birds (Fig. 2). On day 19 p.i., significant reduction was observed in birds fed 10% EYP ($P < 0.005$), and in birds fed 5% EYP ($P < 0.005$) compared with the control, accounting for a mean $4 \log_{10}$ CFU/g and $5 \log_{10}$ CFU/g reduction, respectively. Although a marked decrease in fecal shedding was noticed during this period, variable levels of bacteria were still detectable from the cecal contents at 23 days p.i. in birds fed with 10% and 5% EYP (Table 3). However, a mean reduction of $4.61 \log$ CFU in the livers of 10% EYP-fed ($P \leq 0.005$) and a $5.81 \log$ CFU reduction in 5%-EYP fed birds ($P \leq 0.005$) were observed compared with the livers of the control group. In the cecal contents, the SE levels detected in birds that tested positive were $2.43 \log$ CFU lower in 10% EYP-fed and $2.78 \log$ CFU lower in 5% EYP-fed birds compared with the control group.

A significantly lower inflammation score characterized by the presence of focal areas of hemorrhages in the cecal villi was observed in birds fed 5% EYP, suggestive of a subtle inflammatory change, reflecting the fecal SE clearance (Table 4), whereas at this stage the extent of inflammation in the control birds was severe, characterized by a multifocal coalescing area of hemorrhages and edema. In addition, hypertrophy and hyperplasia of mucous gland cells were observed in the mucosa of the control birds reflecting the extent of SE cecal carriage, whereas moderate hyperplasia was observed in birds fed 10% and 5% EYP. The histological changes observed in birds fed 10% EYP and 5% EYP were similar, but mild to moderate necrosis of the villous tips was evident in birds fed 10% EYP. The differences in histological scores complemented by the SE isolations in 10% EYP-fed and 5% EYP-fed birds further explains the absence of a dose-response relationship. An increased level of food proteins may have deleterious effects in the presence of infection. It has been hypothesized that lectins present in food proteins may bind to mucosal cells or the mucus barrier and thus function as receptors for bacterial glycans and enhance bacterial adhesion to the tissues (20). The histological evaluation aimed to investigate the cellular density and composition in the cecal mucosa and cecal tonsils that might

a) Cecal tonsils of birds fed 5% EYP.



b) Cecal tonsils of control birds

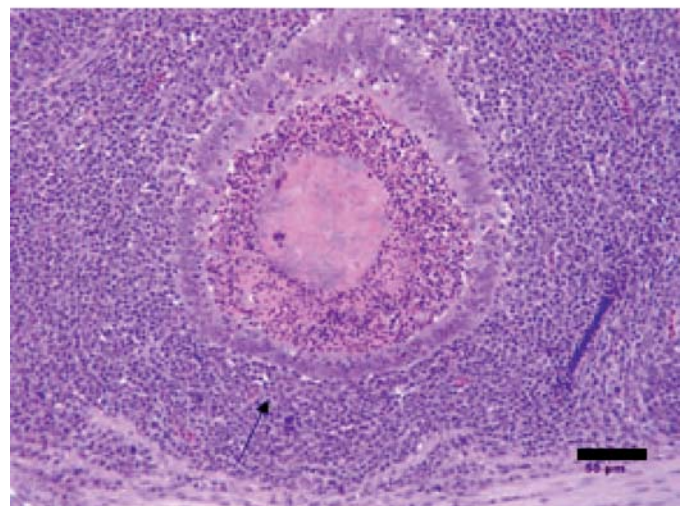


Fig. 3. Photomicrograph of the cecal tonsils of birds. (a) 5% EYP-fed and (b) control birds. Arrows in (a) indicate germinal centers. Arrow in (b) indicates the presence of granuloma. Scale bars indicate $100 \mu\text{m}$ (a) and $50 \mu\text{m}$ (b).

explain the clinical recovery (fecal shedding) of the birds, and not to correlate a direct diet effect. The presence of SE obscured the changes exerted by the diet in the mucosa. Histologically, a significantly increased number of intraepithelial mononuclear cells (mature lymphocytes, plasma cells, macrophages) were observed in the epithelial lining of birds fed 10% EYP or 5% EYP, accompanied by an increased number of lamina propria cells. This suggests that the birds fed EYP exhibited a robust gut-associated lymphoid tissue (GALT) response to restrict the SE and clear the infection. There has been no documented evidence of a direct or indirect immunomodulatory potential of EYP or its derivatives in chickens. Although most of the reports were conducted *in vitro*, a strong body of evidence suggests that various peptides can modulate the immune system-enhancing macrophage functions (2,3,13), which are crucial

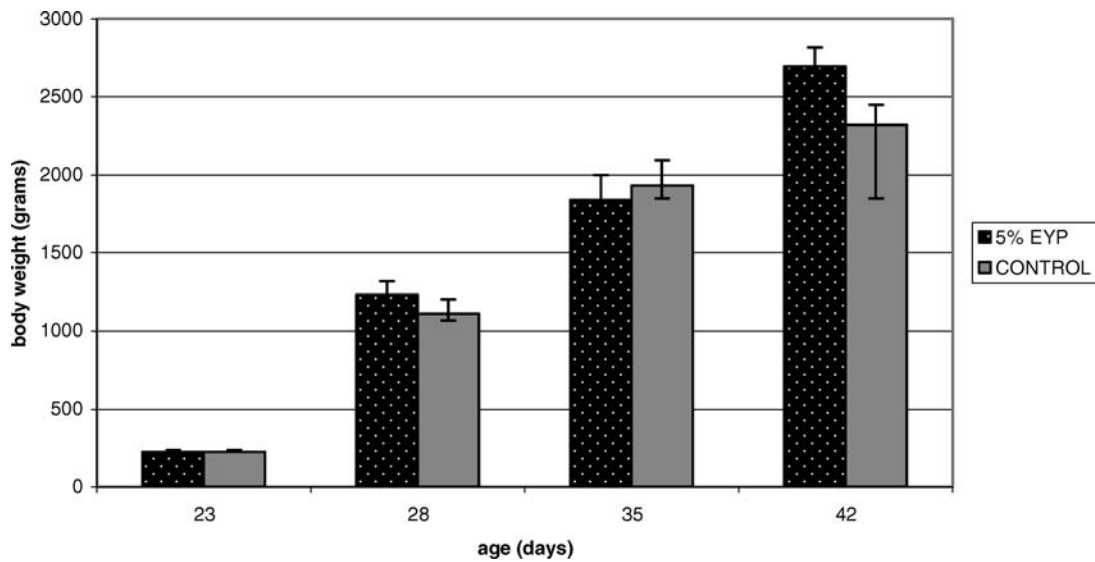


Fig. 4. Average body weights on elimination trial. Vertical bars represent SE. $n = 4-5$. Diet effect: $P = 0.1997$, age effect: $P = 0.0001$, age and diet interaction: $P = 0.0880$.

in the early events of a disease process as pathogen recognition and interaction with the immune system commences (8). In a recent experiment in mice that were unexposed to any pathogen, a diet containing 0.005% egg yolk proteins increased the levels of IgA and IgG B-cell populations (R. Nelson, pers. comm.).

In addition to the observed effects of EYP in the humoral immune system of birds in this trial, it is possible that EYP influenced the activity of other lymphocyte subsets responsible for cell-mediated immunity, partially explaining the observed GALT changes to histology by the presence of mature lymphocytes in the epithelial lining and lamina propria. This is further elucidated by the absence of chronic granulomatous lesions in the cecal tonsils of EYP-fed birds in contrast to birds fed the regular diet (Fig. 3). Such granu-

lomatous lesions as found in the control birds are commonly observed in SE phage type 4 infection (6), indicating a persistent infection and uncontrolled colonization; this event can only be resolved by an active cell-mediated immune response mechanism (21). These histological findings correlate with the negative SE isolations in the liver, ceca, and feces at this stage and are highly suggestive of a well-organized restricted systemic colonization and recovery following intestinal *Salmonella* infection in EYP-fed birds. Additional immunological methods are required to characterize the T-cell population.

Elimination trial. The elimination trial aimed to investigate the eliminative effects of EYP in prechallenged birds. EYP components were recently demonstrated to block the adhesion of

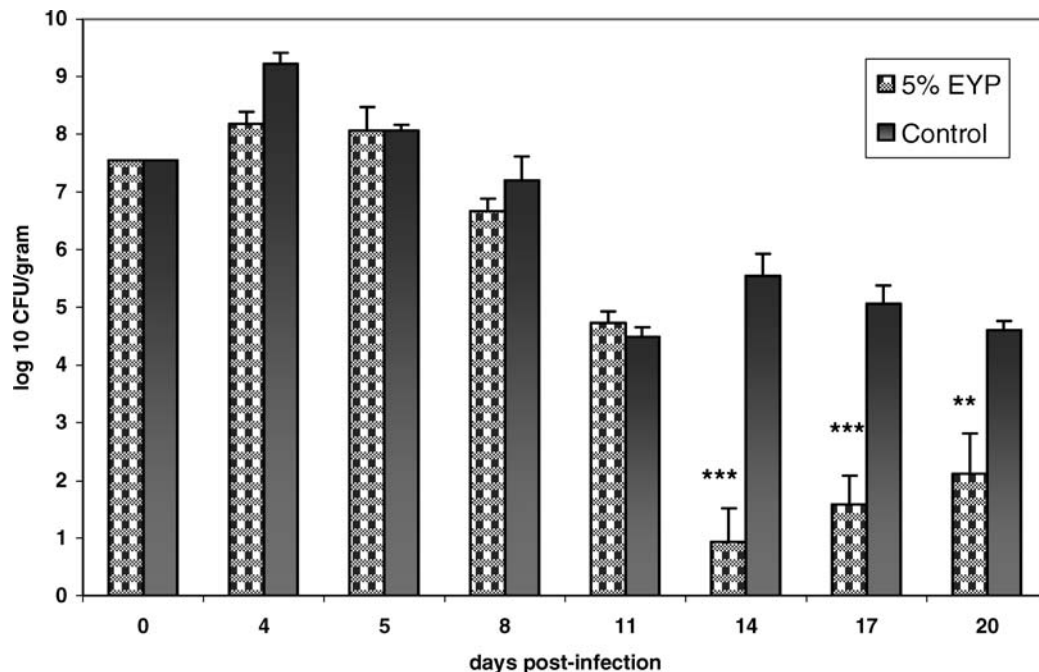


Fig. 5. Fecal shedding on elimination trial. Mean \log_{10} CFU/g \pm SEM. ($n = 6$). *Indicates significant difference with the control ($P \leq 0.05$, Graphpad Instat®). **Indicates very significant difference ($P \leq 0.005$). ***Indicates highly significant difference ($P \leq 0.0005$).

Table 5. Bacterial enumeration in organs; elimination trial.

Organs	Days p.i.				
	1	5	10	17	22
Spleen					
5% EYP	ng ^A	2.33 ± 1.50 ^C (1/4) ^D	1.70 ± 1.09 (1/4)	1.40 ± 0.90 (1/4) ^{***}	0.63 ± 0.39 (1/4) ^{**}
Control	ng	7.32 ± 1.23 (4/5)	2.85 ± 1.15 (2/5)	4.72 ± 0.31 (4/5)	3.55 ± 0.79 (3/4)
Liver					
5% EYP	ng	1.45 ± 0.93 (1/4) ^{***}	3.02 ± 0.73 (3/4)	1.10 ± 0.53 (2/4) ^{***}	1.52 ± 0.92 (2/4) ^{***}
Control	ng	8.32 ± 1.39 (4/5)	3.56 ± 0.09 (5/5)	4.36 ± 0.28(4/5)	6.44 ± 0.42 (4/4)
Cecal wall					
5% EYP	nd ^B	4.75 ± 1.77 (2/4)	4.84 ± 0.59 (4/4)	7.33 ± 0.63 (3/4)	0.65 ± 0.41 (2/4) [*]
Control	nd	6.14 ± 1.30 (4/5)	5.59 ± 0.64 (5/5)	7.63 ± 0.43 (5/5)	2.75 ± 0.59 (3/4)
Cecal contents					
5% EYP	7.43 (2/2)	4.88 ± 1.26 (3/4)	4.14 ± 0.51 (4/4)	5.82 ± 0.55 (4/4) ^{***}	1.75 ± 0.64 (2/4) ^{***}
Control	7.43 (2/2)	4.87 ± 0.52 (5/5)	4.51 + 0.66 (5/5)	8.57 ± 0.86 (5/5)	4.42 ± 0.16 (4/4)

^Ang = no growth.

^Bnd = not determined.

^CMean log₁₀ CFU/g ± SEM.

^DProportion of positives is shown in parentheses.

^{*}Indicates significant difference with the control ($P \leq 0.05$ Graphpad Instat®). ^{**}Indicates very significant difference ($P \leq 0.005$). ^{***}Indicates highly significant difference ($P \leq 0.0005$). All data are triplicate analyses.

pathogens in CaCo2 cells (9). This study is a follow-up trial on the efficacy of the eliminative effect of EYP against SE *in vivo* (10). Ten percent EYP-supplemented diet was not evaluated because of a very small variation in its effects with the 5% EYP in the prevention trial. The body weights are summarized in Fig. 4. The birds fed 5% EYP averaged 2693 g whereas the control birds weighed an average of 2244 g. However, diet effect was not significantly influential ($P = 0.1997$), similar to the prevention trial. The EYP-fed and control birds shed SE as early as 24 hr p.i. (Fig. 5), indicating that SE have proliferated in the gut and that birds were apparently shedding SE prior to feeding with EYP. The feed was introduced after confirmation of SE infection (4 days p.i.) and the shedding was again evaluated after 24 hr of feeding. The EYP-fed and control birds shed SE gradually throughout the course of infection but as early as the 10th day of EYP-feeding, the numbers of SE that was shed in the feces was markedly reduced compared with the control, with a 4.61 log₁₀ CFU ($P \leq 0.0005$) reduction compared with the control group. At 14 day p.i. and thereafter, the variations in shedding between the EYP-fed group and the control group were significantly lower ($P \leq 0.05$).

Initially high levels of SE were detected in the cecal contents of birds in both groups at 24 hr p.i. (Table 5). At 17 and 22 days p.i., a highly significant reduction in SE cecal carriage ($P \leq 0.005$) was observed. However, the levels of SE in the cecal contents did not

reflect the numbers of SE that colonized the cecal wall and significant reduction ($P \leq 0.05$) was observed only on the last testing date (2.10 log₁₀ CFU/g lower). A controlled systemic colonization was observed in birds fed with EYP. A marked reduction of SE was detected in the spleen ($P \leq 0.005$) and liver ($P \leq 0.005$) by 17 days p.i. (last day of feeding) and 22 days p.i. (after feed was withdrawn for 4 days).

The histological changes in the cecal mucosa and cecal tonsils (Table 6) reflected an apparent inflammation and mucosal repair and also suggested that the infection had not completely resolved, confirming SE bacterial isolations in the cecal tissue. An extensive multifocal hemorrhage and edema were observed in the villi flanking the cecal tonsils of birds fed the regular diet. EYP-fed birds, on the other hand, exhibited focal mild hemorrhages, indicating subtle inflammation. This histological finding further demonstrated the protective effect of EYP and a possible reduction of the number of SE organisms in the cecal tonsils at this stage of infection. A restrained systemic colonization indicates that the mucosal barrier has been protected somehow by other mechanisms, such as the following: 1) feeding of EYP to infected birds at 4 days p.i. might have resulted in direct interaction of the EYP components with SE in the gut lumen, thereby preventing further colonization (antiadhesive effects) and exclusion of SE, and/or 2) EYP components that have reached the phagocytes (macrophages and heterophils) might have influenced phagocytic activity and intracellular killing of SE (immunomodulatory properties), resulting in a reduced systemic dissemination of the organism, as explained previously. Although at less magnitude on the overall effect of EYP in broiler birds, the elimination trial supports previous studies in layers suggesting (10) that EYP is an efficient alternative to antimicrobials and, in the face of an SE infection, can be used as a feed supplement to reduce SE.

Table 6. Histopathological examination at 23 days p.i.; elimination trial. Values in parentheses indicate average histopathological scores.

Parameter	5% EYP	Control
Inflammation	Mild (1.00) [*]	Moderate (2.00)
Mucous gland cell hyperplasia	Moderate (2.00)	Moderate (2.00)
Intraepithelial mononuclear cells	Moderate (2.00)	Moderate (2.00)
Lamina proprial cell population	Moderate (2.00)	Moderate (2.00)
Other lesions:		
Necrosis of villous tips	Mild to moderate	Mild to moderate
Granuloma (cecal tonsils)	None	None

^{*}Indicates significant difference with the control ($P \leq 0.05$, Kruskal-Wallis for nonparametric analysis; Graphpad Instat®).

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