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Efficacy of Prebiotics, Probiotics, and Synbiotics on Laying Hens and Broilers Challenged with *Salmonella* Enteritidis

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This study aimed to evaluate the efficacy of dietary prebiotic, probiotic, and synbiotic products for controlling infection in laying hens and broiler chickens challenged with *Salmonella enterica* serovar Enteritidis (SE). These products could replace the use of antibiotics, which would avoid the problem of hastening antimicrobial resistance for both types of birds. *Salmonella*-free 1-day-old (1-d-old) layers chicks and broilers chicks were inoculated with SE resistant to nalidixic acid and spectinomycin (SE Nal^r Spec^r) and divided into four groups: 1) control (without feed additives); 2) probiotic (*Bacillus subtilis*, *Lactobacillus acidophilus, L. casei, Enterococcus faecium, Bifidobacterium longum*); 3) prebiotic (inulin, fructooligosaccharide, mannanoligosaccharide, and oligosaccharide); and 4) synbiotic (85% of the probiotic＋15% of the prebiotic additives). The presence of SE Nal^r Spec^r in cloacal swabs was analyzed at 7, 14, and 21 days post-infection (dpi) in laying hens and broilers. The number of SE Nal^r Spec^r per gram of cecal contents was determined at 7, 14, and 21 dpi in laying hens and at 2, 5, 7, 14, and 21 dpi in broilers. The results showed that the prebiotic additive reduced the occurrence of SE in cloacal swabs from laying hens but not from broilers. In the groups of laying hens and broilers that received prebiotics, the isolation and counts of SE Nal^r Spec^r were lower during the first week post-infection but not throughout the experiment. The probiotic and synbiotic additives did not influence the SE infection in laying hens and broilers; in contrast prebiotics had a protective effect during the first week post-infection.

Key words: additives, chicken, pathogenic bacteria, *Salmonella*-free

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Introduction

Salmonella spp. are one of the most frequently reported pathogenic bacteria found in the food production chain that affect human health (Mead *et al*., 1999), and therefore are frequently the subject of food safety policies and interventions (Scallan *et al*., 2011). Investigations into outbreaks and sporadic cases have repeatedly indicated that when a food vehicle is identified, the most common sources of *S. enterica* infection are poultry and poultry products, especially in cases of outbreaks from undercooked and raw eggs (Velge *et al*., 2005).

Once introduced onto farms, enteric pathogens such as *S. enterica* serovar Enteritidis (SE) easily becomes disseminated among animals (Freitas Neto *et al*., 2010). To prevent potential food borne infections caused by *Salmonella* spp. in humans, it is imperative to perform an effective inspection of the food production chain. The infection of birds may occur orally or vertically, in which a contaminated egg produces a naturally infected chick (Desmidt *et al*., 1998). In the case of meat production, enteropathogenic organisms might contaminate the carcasses during slaughter and evisceration, representing another possible transmission route for these agents to infect humans (Uyttendaele *et al*., 1998).

The use of prebiotic, probiotic and synbiotic additives for pathogen control and performance enhancement in poultry production has gained attention recently due to the increasing restriction of antibiotics as growth-promoting agents (Gaggia *et al*., 2010). According to Van Immerseel *et al*. (2009), the prophylactic and curative use of antibiotics to control *Salmonella* is not recommended for three reasons: 1) antibiotic-resistant *Salmonella* (and other) strains have emerged; 2) there is a concern about the presence of antibiotic residues

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in meat and 3) most antibiotics fail to eliminate *Salmonella* from animals, although some decreased contamination from this pathogen in animals has been observed.

Prebiotics are compounds that are unavailable to, or indigestible by, the host animal, but are available to a specific proportion of the microbial population. They are often described as functional foods or nutraceuticals (Schrezenmeir and De Vrese, 2001). Probiotics are products that exert beneficial health effects in the host. They are viable, defined microorganisms in sufficient numbers to alter the microflora by implantation or colonization in a compartment of the host (Schrezenmeir and De Vrese, 2001). Products containing both prebiotics and probiotics are known as synbiotics, and this term should be reserved for products in which the prebiotic compound selectively favors the probiotic compound (Schrezenmeir and De Vrese, 2001). The aim of this study was to evaluate the effect of a dietary supplementation containing either prebiotic, probiotic or synbiotic products on laying hens and broilers challenged with *S.* Enteritidis.

Materials and Methods

Bacteria and Inocula

The bacteria used in the experiments was a spontaneous mutant of SE resistant to both nalidixic acid and spectinomycin (SE Nal^r $Spec^r$), maintained by the Avian Diseases Laboratory of FCAV-UNESP, campus (Jaboticabal, Brazil). SE Nal^r $Spec^r$ cultures were grown in Luria–Bertani broth and incubated overnight in a shaking incubator (100 revolutions/min) at 37℃. This culture contained approximately 10^9 colony-forming units/mL (CFU/mL).

Experimental Procedure

Two types of birds were used: commercial layers of the brown variety (experiment 1) and broilers from a commercial hatchery (experiment 2). They were obtained at 1-d-old from commercial hatcheries. A total of 120 birds were used in each experiment. All birds received 0.1 mL from a culture containing 10^9 CFU/mL SE Nal^r $Spec^r$ by gavage at 1-d-old, and then were randomly distributed into four groups. 10 birds per treatment group in experiment 1, and 6 birds per treatment group in experiment 2, were analyzed on different occasions. Groups were caged in a room under controlled environmental management (27±2℃ with a 12 h light/dark cycle). The feed composition and the experimental treatments are provided in Tables 1 and 2, respectively. The birds had unrestricted access to water and feed *ad libitum*. In both experiments, cloacal swabs were taken at 7, 14, and 21 days post-infection (dpi). To collect the cecal contents, the birds were killed by cervical dislocation in experiment 1 at 7, 14, and 21 dpi and in experiment 2 at 2, 5, 7, 14, and 21 dpi.

On arrival, the transport chick boxes were tested to ensure their *Salmonella*-free status (Zancan *et al*., 2000) and all boxes were determined to be *Salmonella*-free.

The feeding trial was conducted under the approval of the Ethics Committee of Paulista State University "Julio de Mesquita Filho" (Process # 014143/12).

Bacteriological Analyses

Bacteriological analyses were carried out as described by Barrow and Lovell (1991) with some modification. Briefly, cloacal swabs were placed in selenite broth containing novobiocin (40 μg/mL) (SN) and directly placed onto Brilliant Green Agar with nalidixic acid (25 μg/mL) and spectinomycin (100 μg/mL) (BGA Nal/Spec). The cultures were incubated at 37℃ for 24 h. In the absence of growth, the appropriate enriched swab cultures were inoculated onto fresh BGA Nal/Spec plates.

After the harvesting of cecal contents, the samples were weighed and serially diluted (1:10) in phosphate-buffered

Table 1. **Basal composition and nutrient content per 100 kilogram of broiler diets**

Ingredient	Quantity (kg)
Ground maize	66.6
Soybean meal	29.7
Dicalcium phosphate	1.69
Lime stone powder	1.07
Sodium chloride	0.45
Methionine	0.09
Polimix[1]	0.40

[1] Polimix provided the following: vitamin A, 10000000 IU; vitamin D_3, 2000000 IU; vitamin E, 8000 mg; vitamin B_1, 1200 mg; vitamin B_2, 4500 mg; vitamin B_6, 1300 mg; vitamin B_{12}, 8000 mcg; folic acid, 300 mg; biotin, 52 mg; niacin, 30000 mg; calcium pantothenate, 11000 mg; Co, 6000 mg; Cu, 100 mg; I, 1000 mg; Fe, 50000 mg; Mn, 60000 mg; Zn, 40000 mg; Se, 200 mg; antioxidant, 1300 mg per kilogram of the Polimix product.

Table 2. **Composition of the feed additives**

Group	Composition	Concentration (additive g/kg feed)
1) Control	—	—
2) Probiotic	*Bacillus subtilis, Lactobacillus acidophilus, L. casei, Enterococcus faecium, Bifidobacterium longum* at the same quantitative proportion	0.85
3) Prebiotic	inulin, fructooligosaccharide, mannanoligosaccharide, and oligosaccharide at the same quantitative proportion	0.15
4) Synbiotic	85% of the probiotic+15% of the prebiotic additive	1.0

saline (PBS) with pH 7.4. Viability counts for the SE Nal^r Spec^r in the samples were measured by plating aliquots of the serial dilutions on BGA Nal/Spec and incubating the plate at 37℃ for 24 h. The first dilution was added to an equal volume of double-strength SN. This dilution was incubated at 37℃ overnight and plated on BGA Nal/Spec. No growth of SE was detected on the viability count assay.

Statistical Analyses

Cloacal swabs were analyzed with the chi-square test to determine significant differences among treatments for SE incidences. The means of Log_{10} viable bacterial counts from cecal contents were submitted to one-way ANOVA. The means were compared using Tukey's multiple comparison test to verify the protective effect of the additives. Significant differences were assessed at the probability level of $P<0.05$.

Results and Discussion

A number of feed additives have gained commercial acceptance for reducing *Salmonella* (Berge and Wierup, 2012). In this study, three feed additives were tested– prebiotics, probiotics, and synbiotics– by adding them to the rations fed to laying hens and broilers inoculated with *S.* Enteritidis. Table 3 shows the results of the cloacal swab analyses from laying hens and broilers at 7, 14 and 21 dpi, with a total of 18 observations for each treatment. In laying hens, a significant difference was observed between the prebiotic and the control treatment, while the probiotic and synbiotic treatments did not show any effect on the presence of SE. In broilers, the prebiotic, probiotic, and synbiotic treatments did not demonstrate significant differences compared with the control treatment. The detection of SE, in swab and cecal content count assays, was higher in broilers than in hens (Tables 4 and 5). This difference in SE recovery between hens and broilers most likely occurred because there were differences in immune responses eventually promoted by growth rates. These findings were in agreement with Parmentier *et al*. (2010), who suggested that a higher body weight in broilers negatively affected the immune humoral response, while the genetic changes of layers toward egg production had less negative impact on the birds' immune systems. In addition, a more consistent and permanent immune response has been observed in laying hens compared to broilers (Koenen *et al*., 2002).

Table 4 shows a significant reduction in the SE count in laying hens treated with the prebiotic additive at 7 dpi. A significant reduction was also observed at 5 dpi in broilers receiving prebiotic feed (Table 5). At 14 and 21 dpi, no significant differences were observed in laying hens or broiler chickens fed the prebiotic diet. The prebiotic additive was the only treatment that had effect in reducing SE counts, because it acted directly on existing host microorganisms. According to Figueroa-Gonzales *et al*. (2011), the prebiotics conferred specific changes in the composition and/or activity of the native gastrointestinal microbiota. These changes could be associated with the environment as pH values, competition for nutrients and direct antagonists effects that inhibit growth of some pathogenic microorganisms as suggested by Collins and Gibson (1999). The prebiotics used in this study reduced SE recovery in challenged neonatal layer chicks and broilers chicks in the first week.

The prebiotic product tested was a mix of different compounds, including inulin, fructooligosaccharide, mannanoligosaccharide, and oligosaccharide. The prebiotic effect of these substances has been evaluated individually by different authors corroborating with the results reported here. For example, Bailey *et al*. (1991) and Fukata *et al*. (1999) reported that the inclusion of oligofructose in the chicks' diets enabled a substantial reduction of *Salmonella* colonization in the gastrointestinal tract. Nabizadeh (2012) showed that inulin supplementation caused no significant differences in the microfloral counts of the ileal contents but significantly increased the *Bifidobacteria* counts and decreased the *E. coli* counts of the cecal contents. Baurhoo *et al*. (2007) pointed

Table 3. **The presence *Salmonella* Enteritidis in cloacal swabs analyzed at 7, 14, and 21 days post-infection (dpi) from laying hens (experiment 1) and broilers (experiment 2) fed rations with either prebiotic, synbiotic or probiotic additives**

Experiments	Treatments				
	Dpi	Control	Prebiotic	Synbiotic	Probiotic
1	7	4/6	1/6	3/6	1/6
	14	3/6	0/6	1/6	1/6
	21	0/6	0/6	0/6	0/6
	P/T	7/18^a	1/18^b	4/18^a	2/18^a
2	7	5/6	5/6	6/6	6/6
	14	0/6	2/6	3/6	4/6
	21	4/6	0/6	0/6	0/6
	P/T	9/18^a	7/18^a	9/18^a	10/18^a

P/T: number of SE-positive birds (P) out of a total of 18 observations (T).
* Common superscript letters do not differ significantly at $P<0.05$ according to chi-square test.

Table 4. **Experiment 1 cecal results from laying hens showing the viable number (log_{10}) of SE Nal^r $Spec^r$ bacteria in the cecal contents analyzed at 7, 14, and 21 days post-infection**

Treatment	Log_{10} viable number of SE Nal^r $Spec^r$ per gram of cecal contents		
	7 dpi	14 dpi	21 dpi
Control	*1.59[a]** (N–7.87)	0.67[b] (N–2.70)	0.20 (N–2.00)
Prebiotic	0.60[b] (N–2.00)	0.28[b] (N–2.78)	N (N–N)
Synbiotic	0.80[ab] (N–8.00)	1.40[a] (N–2.00)	0.2 (N–2.00)
Probiotic	1.66[a] (N–2.60)	N[b] (N–N)	0.2 (N–2.00)

N: log_{10} <2.0.
Values in parentheses represent the range of viable log_{10} counts of SE Nal^r $Spec^r$.
* The median count per gram from 10 birds is provided.
** Values within groups that do not share common superscript letters differ significantly at $P<0.05$ according to Tukey's test.

Table 5. **Experiment 2 cecal results from broilers showing the viable number (log_{10}) of SE Nal^r $Spec^r$ bacteria in the cecal contents analyzed at 5, 7, 14, and 21 days post-infection**

Treatment	Log_{10} viable number of Nal^r $Spec^r$ per gram of cecal content				
	2 dpi	5 dpi	7 dpi	14 dpi	21 dpi
Control	4.87 (2.00–6.95)	6.20[a] (4.48–7.63)	6.32 (5.04–7.30)	1.01 (N–6.08)	N (N–N)
Prebiotic	5.39 (4.28–7.25)	1.16[b] (N–6.95)	3.72 (N–7.40)	0.51 (N–3.06)	N (N–N)
Synbiotic	3.56 (N–6.25)	4.30[a] (N–7.36)	5.72 (4.18–7.52)	2.00 (N–2.00)	N (N–N)
Probiotic	3.75 (2.00–5.70)	5.35[a] (N–7.34)	4.70 (N–7.66)	0.51 (N–3.07)	N (N–N)

N: log_{10} <2.0.
Values in parentheses represent the range of viable log_{10} counts of SE Nal^r $Spec^r$.
* The median count per gram from 6 birds is provided.
** Values within groups that do not share common superscript letters differ significantly at $P<0.05$ according to Tukey's test.

out that the mannanoligosaccharides act by blocking the sites of bacterial adhesion, thus reducing the binding capacity of some pathogenic bacteria in the intestinal mucosa. Thus, the mix of different prebiotics could be advantageous in reducing the number of SE.

Our finding that supplementation with probiotics or synbiotics had no effect on *Salmonella* reduction was in agreement with Ribeiro *et al*. (2007), but differed from the results reported by Higgins *et al*. (2005) and Wolfenden *et al*. (2007), who demonstrated a beneficial effect of a probiotic in poultry with *Salmonella* infections. The experiments related to these products are somewhat controversial. For example, Berge and Wierup (2012) recently reported that the challenges with nutritional interventions for *Salmonella* control were variable depending on the nutritional management and *Salmonella* status of the flock. Both probiotic and synbiotic use had limited efficacy on decreasing SE colonization although it was not certain that the microorganisms present in these products failed to colonize the enteric microenvironment. Furthermore, it is necessary to consider the composition of the commercial products, their dosage, the route of administration (by feed or water) and the farm sanitary conditions. All these factors are able to influence the efficacy of the products.

Conclusion

The results obtained showed that prebiotics seem to confer a protective effect in chicks during the first days post-infection, while the probiotics and synbiotics tested did not influence SE infection in laying hens and broilers.

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