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Organic acids and/or compound with defined microorganisms to control *Salmonella enterica* serovar Enteritidis experimental infection in chickens

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ABSTRACT

The association of human foodborne salmonellosis with poultry products enhanced the efforts to control *Salmonella* Enteritidis in poultry farms. Dietary organic acid supplementation is one of the measures currently used to reduce the presence of undesirable microorganisms. Another method to control enteric *Salmonella* in poultry is competitive exclusion using defined or undefined microorganisms products. Organic acids and microbiological methods to accelerate the development of the intestinal microbiota can be used individually or in combination. The present study evaluated the effect of dietary supplementation of an acidifier and of a defined multi-strain microbial mixture (Biomin® PoultryStar) via drinking water in the control of the intestinal colonization of broilers by *Salmonella* Enteritidis. Four experiments were performed. The first experiment showed that the organic acids mixture was able to prevent *Salmonella* Enteritidis colonization of ceca in both inclusion rates applied ($p<0.05$). In the second and third experiments the probiotic either individually or in combination the acidifier, both in high and low doses reduced the incidence of *Salmonella* Enteritidis in the cecal contents ($p<0.05$). In these three experiments, birds were orally challenged. Similar results were obtained in a fourth trial, in which challenge was made by contact.

INTRODUCTION

The control of *Salmonella* in commercial poultry was necessary for the development of poultry industry. Rearing birds in high densities, living in close contact with feces allows the infection and the dissemination of pathogens. Therefore, pathogenic bacteria associated to human foodborne diseases must be controlled. In the beginning of the 1980s worldwide outbreaks of human salmonellosis caused by *Salmonella* Enteritidis were linked to the consumption of poultry products, leading to a reinforcement of measures to control *Salmonella* in broilers (Wray & Davies, 1994), which started in Brazil around 1990 and was responsible for a plan to control avian diseases in the Brazilian poultry industry (PNSA) (Brasil, 2003).

One of the control measures was the inclusion of organic acids into the feed, aiming at reducing the number of undesirable microorganisms. Organic acids inhibit bacterial growth by decreasing intestinal pH (Barcellos *et al.,* 2004), interfere with bacterial metabolism by decreasing the cytoplasmatic pH, as well as inhibit enzymatic action and DNA synthesis (Vieira & Viola, 2004). However, organic acid compounds do not cause residues in meat, and therefore are not harmful to human beings. The mode of action of organic acids on infectious pathogenic microorganisms was well documented by Cherrington *et al.* (1991). A blend of formic acid and propionic acid included in the feed can prevent

cecal colonization of newly-hatched chicks by *Salmonella enterica* serovars Typhimurium, Enteritidis, Agona and Infantis (Iba & Berchieri Jr., 1995). This is consistent with previous findings by Hinton & Linton (1988), who reported that a blend of organic acids did not completely eliminate *Salmonella* from treated feed, but when this feed was given to the birds, there was no cecal colonization by *Salmonella*. According to Cherrington *et al.* (1991), the full action of organic acids is expressed in the crop due to its high moistures content humidity. However, according to Oliveira *et al.* (2000), the contamination by contact between infected and non-infected birds cannot be controlled by organic acids.

Another way to control enteric *Salmonella* in poultry is competitive exclusion, using defined or undefined microorganisms mixtures. This procedure was described initially by Nurmi & Rantala (1973), and was later supported by several studies conducted worldwide (Ziprin *et al.*, 1993; Mead, 2000; Schneitz, 2005). Undefined cultures usually showed better performance as compared to products containing a defined culture of microorganisms (Hinton & Mead, 1991). The competitive exclusion effect is apparently due to the competition for sites of adherence, and to a decrease in cecal pH through the production of short chain volatile organic acids (Ziprin *et al.,* 1991). Although a defined culture of microorganisms is indicated to prevent enteric colonization by *Salmonella*, this method is controversial. The bacteria present in the culture can improve the composition of the intestinal flora (Santos & Turnes, 2005), by improving the quality of the intestinal villi (Luqueti *et al,* 2005). This may allow the control of enteric pathogenic bacteria without the use of the antimicrobial drugs (Santos & Turnes, 2005).

One of the main sources of *Salmonella* infections in poultry farms is the feed. For this reason, efforts have been made to improve the microbiological quality of feed by adding organic acids to the feed, and by offering competitive exclusion products to the newly-hatched chicks in order to accelerate of the establishment of the microflora. These two classes of products can be used together, with no influence on each other, as demonstrated by Hinton *et al.* (1991) and Oliveira *et al.* (2000).

This study aimed at evaluating the effect of a commercial blend of organic acids (acidifier) and a well-defined multi-strain probiotic product, containing microorganisms to colonize the gut of day-old chicks in the intestinal colonization by *Salmonella*. The acidifier contained formic acid and propionic acid. The defined microbial product included following probiotic strains: *Enterococcus* sp., *Pediococcus* sp., *Bifidobacterium* sp. and *Lactobacillus* spp, which were isolated from different parts of the gastrointestinal tract of broilers and selected for their ability to establish a healthy gut.

MATERIAL AND METHODS

a) Bacterium challenge

A spontaneous mutant of the *Salmonella enterica* serovar Enteritidis, resistant to nalidixic acid and spectinomycin (SE Nal^rSpc^r), kept by the laboratory of avian diseases of the FCAV-Unesp, was used. Broth culture was prepared in nutrient broth (Oxoid CM 67) incubated at 37°C for 18 hours in a shaking incubator. The culture contained between 1.3 and 3.3 X 10^9 CFU/mL.

This overnight broth culture of SE Nal^rSpec^r was diluted 1000 times in fresh nutrient broth. Birds were challenged either by inoculating 0.1mL directly inoculated into the crop of 3-day-old chicks, or by contact, placing two infected birds in each box, which housed 9 uninfected birds.

b) Inclusion of acidifier into feed

The acidifier was included at 1.5 kg per ton or 3.0 kg per ton of feed.

c) Inclusion of defined probiotic product

During the first three days of the chicks' life, the multi-strain probiotic (Biomin® PoultryStar, Biomin GmbH, Austria) was daily added to the drinking water at a concentration of 20 g per 1,000 birds.

d) Birds

Day-old broiler chicks were provided by a commercial hatchery. At arrival, chicks they were inspected to ensure they were free from *Salmonella*. Drag swabs were taken from inside the transport boxes, and blood from several birds for serological examination. Swabs were placed in a flask containing Selenite broth (Oxoid CM 395 + L121) plus novobiocin, and incubated at 37° C overnight before plating on brilliant green agar, and then again incubated at 37° C overnight (Zancan *et al.*, 2000; Gama *et al.*, 2003). Serum samples were tested by slide agglutination test with *Salmonella* colonies grown on nutrient agar.

Birds were placed in netted wooden boxes, which were previously disinfected. Boxes were equipped with a heating source, water and commercial starter feed

with no antimicrobial drugs were offered *ad libitum*. There were nine birds per box, with two boxes per experimental group. When birds were challenged by contact, two additional seeder birds (experiment 4) were placed in each pen.

e) Bacterial enumeration

The method of bacterium enumeration was performed according to Barrow *et al.* (1987). Decimal dilutions of the cecal contents were prepared using PBS pH 7.4 (saline buffer), and bacteria were counted on brilliant green agar plates (Oxoid CM 263), containing sodium nalidixate (100 mg/mL) and spectinomycin (100 mg/mL). The plates were incubated at 37° C for 24 hours. The results (CFU/g) were transformed in $\log_{10}$, and submitted to analysis of variance. Means were compared by the test of Tukey ($p < 0.05$; SAS, 2002).

f) Experimental design

Four experiments were performed. Nine birds per replicate, 2 replicates per group.

Experiment 1

Two batches of feed were treated with acidifier, one containing 1.5 kg per ton of feed (group A), and the other 3.0 kg per ton (group B). A third batch did not include any organic acids blend (group C).

Birds were challenged via inoculation into the crop at thre days of age. At 5, 7, and 10 days of age three birds were sacrificed, and their cecal contents were examined to estimate viable SE Nal^{r}Spc^r counts.

Experiment 2

This experiment included four groups:

Group A – feed containing 3.0 kg acidifier per ton of feed
Group B – feed containing 3.0 kg acidifier per ton of feed, and probiotic added to the drinking water
Group C – drinking water with probiotic
Group D – no additives

SE Nal^{r}Spc^r challenge and counting were carried out as described in experiment 1.

Experiment 3

This experiment included three groups:

Group A – drinking water with probiotic
Group B – feed containing 1.5 kg acidifier per ton of feed, and probiotic added to the drinking water
Group C – no additives

SE Nal^{r}Spc^r challenge and counting were carried out as described in experiment 1.

Experiment 4

This experiment was carried out as experiment 3, but instead of individually inoculating birds, two infected birds were placed inside each box on the third day of life of the newly-hatched chicks.

RESULTS

The inspection of the birds and the transport boxes at arrival did not show any evidence of *Salmonella* sp.

The first experiment was carried out to assess the effect of two concentrations of the feed acidifier on the prevention of cecal colonization by SE Nal^{r}Spc^r. As shown in Table 1, both inclusion rates used were able to reduce viable SE Nal^{r}Spc^r counts; however, the best results were obtained when the feed contained 3.0 kg of the product per ton ($p<0.05$).

In the second experiment (Table 2), the effect of the feed acidifier (3kg/ton of feed) and the probiotic added to the drinking water was simultaneously evaluated. The results showed that either the acidifier or probiotic alone, or both products together prevented the cecal colonization by SE Nal^{r}Spc^r ($p< .05$).

The third experiment (Table 3) was performed to assess the effect of the defined multi-strain probiotic product alone, and of a combination of the acidifier at a lower inclusion rate (1.5kg/ton) and the probiotic . The results indicated that all treatments prevented cecal colonization by SE Nal^{r}Spc^r ($p<0.05$).

Due to the interesting results obtained in the previous three experiments with individually infected birds, a fourth trial was carried out to check if the same results could be obtained if contact the infection was promoted by the contact of infected with non-infected birds. The results in Table 4 show that there was a decrease in cecal colonization by SE Nal^{r}Spc^r with the combination of products, but the best results were obtained when the defined multi-strain probiotic product was used alone.

DISCUSSION

The association of poultry salmonellosis to human foodborne disease put pressure on the efforts to control *Salmonella* Enteritidis in poultry farms. The use of

Table 1 – Viable SE Nal^{r}Spc^r counts in the cecal contents of broilers receiving feed containing acidifier (Experiment 1).

Group	Treatment	Log_{10} viable number of SE Nal/Spec per gram of cecal contents
A	ACIDIFIER (1.5 Kg/ton)	4.59# (2.75 - 6.33) ab*
B	ACIDIFIER (3.0 Kg/ton)	3.47 (N - 5.25) a
C	No additive (Control)	5.06 (3.45 - 6.67) b

Viable counts are expressed as mean from 18 birds (range in parenthesis). N = Log_{10} < 2. * Means followed by different letters are significantly different (Tukey, p <0.05).

Table 2 – Viable SE Nal^{r}Spc^r counts in the cecal contents of broilers receiving feed containing acidifier or/and probiotic.

Group	Treatment	Log_{10} viable number of SE Nal/Spec per gram of cecal contents
A	ACIDIFIER (3.0 kg/ton)	2.43#(N-3.68) a*
B	ACIDIFIER (3.0 kg/ton)Biomin® PoultryStar (20g/1000 birds)	N (N-N) a
C	Biomin® PoultryStar (20g/1000 birds)	N (N-N) a
D	No feed additive (Control)	3.62 (N-5.53) b

Viable counts are expressed as mean from 18 birds (range in parenthesis). N = Log_{10} < 2. * Means followed by different letters are significantly different (Tukey, p <0.05).

Table 3 - Viable SE Nal^{r}Spc^r counts in the cecal contents of broilers receiving feed containing acidifier or/and probiotic.

Group	Treatment	Log_{10} viable number of SE Nal/Spec per gram of cecal contents
A	Biomin® PoultryStar (20g/1000 birds)	2.90# (N - 4.47) a*
B	ACIDIFIER (1.5 kg/ton)Biomin® PoultryStar (20g/1000 birds)	2.96 (N - 4.59) a
C	Control	4.10 (2.67 - 5.53) b

Viable counts are expressed as mean from 18 birds (range in parenthesis). N = Log_{10} < 2. * Means followed by different letters are significantly different (Tukey, p <0.05).

Table 4 - Viable SE Nal^{r}Spc^r counts (log_{10}) in the cecal contents of broilers receiving feed containing acidifier or/and probiotic, challenged by contact birds

Grupo	Treatment	Log_{10} viable number of SE Nal/Spec per gram of cecal contents
A	Biomin® PoultryStar (20g/1000 birds)	2,75# (N - 3,87) a*
B	ACIDIFIER (1.5 kg/ton) Biomin® PoultryStar (20g/1000 birds)	3,48 (N - 5,26) ab
C	Control	4,01 (2,67 - 5,68) b

Viable counts are expressed as mean from 18 birds (range in parenthesis). N = Log_{10} < 2. * Means followed by different letters are significantly different (Tukey, p <0.05).

organic acids and microbiological methods, alone or together, to accelerate the development of the intestinal microbiota has been suggested (Hinton *et al.*, 1991; Oliveira *et al.*, 2000). Therefore, the present study aimed at assessing the effects of the feed inclusion of an acidifier and the administration of a defined multi-strain microbial product (Biomin® PoultryStar) via drinking water on the control of the intestinal colonization by *Salmonella* Enteritidis in broilers.

According to the data shown in Table 1, the acidifier was able to prevent cecal colonization by *Salmonella* Enteritidis in both applied inclusion rates (p<0.05), but the best better results were obtained when at the higher inclusion rate.

Based on these results, a second experiment was performed, now also including the acidifier at a higher inclusion rate (3 kg/ton). The results exhibited in Table 2 show that the probiotic product either alone, or with the acidifier, was able to reduce the presence of *Salmonella* Enteritidis in the cecal contents. Although the products assessed here are not the same as used in previous investigations, the results are consistent with those of previous studies, in which organic acid compounds did not interfere with the action of products for the establishment of the intestinal microbiota (Hinton *et al.*, 1991; Oliveira *et al.*, 2000). Hume *et al.* (1993) reported an improvement in the control of *Salmonella* in the intestinal tract by the concomitant use of organic acids and competitive exclusion products.

In the third experiment (Table 3), the association of both products also included a lower low dose of the acidifier (1.5 kg/ton). Again, cecal colonization by *Salmonella* was prevented (p<0.05). Similar results were obtained in the fourth experiment carried out also with both products together (Table 4), but at this time, the challenge was done by contact (p<0.05).

CONCLUSIONS

Based on these results, we conclude that the well-defined multi-strain probiotic product (Biomin® PoultryStar), offered via drinking water, controls the intestinal colonization of chickens by *Salmonella* Enteritidis.

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