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Effect of an *Aspergillus* Meal Prebiotic on *Salmonella* Infection in Turkeys and Broiler Chickens

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Abstract: The objective of this study was to evaluate the effect of 0.2% dietary *Aspergillus* Meal (AM) against horizontal transmission of *Salmonella* sp. in turkeys and chickens. Experiment 1 evaluated the effect of AM against horizontal transmission of *Salmonella* Enteritidis (SE) in turkeys. Day-of-hatch turkeys were assigned to untreated control or AM prebiotic-fed groups. Five additional seeder turkeys per group were challenged with 1.5×10^5 cfu SE and placed in each of the treatment groups 24 h later. At ten, twenty and thirty days of age, Cecal Tonsils (CT) were cultured for SE recovery. A significant reduction in SE recovery (25%, 30% and 35% respectively) was observed in prebiotic-fed turkeys when compared with controls ($p < 0.05$). In experiments 2 and 3, the effect of AM against horizontal transmission of *Salmonella* Typhimurium (ST) in chickens was evaluated. In each experiment, day-of-hatch chickens were assigned to untreated control or AM prebiotic-fed groups. Five additional seeder chicks per group were challenged with 1.25×10^5 cfu of ST and placed in each of the treatment groups 24 h later. At ten days of age, Liver/Spleen (L/S) and CT were cultured for ST recovery. In experiments 2 and 3, percent reduction of ST from L/S and CT were 60%, 75% and 55%, 60% respectively when compared to non-treated controls. These results suggest that the addition of AM as a prebiotic at 0.2% may have a beneficial effect in reducing *Salmonella* levels and may enhance overall food safety of poultry meat.

Key words: *Aspergillus* sp., prebiotic, turkeys, chickens, food safety

INTRODUCTION

Poultry products have been associated frequently with the transmission of enteropathogens, such as *Salmonella* (Cox and Pavic, 2010). Although *Salmonella enterica* serovars are some of the most extensively studied bacterial pathogens, much research is still needed (Boyle *et al.*, 2007). Researchers worldwide have been working on sustainable alternatives due to the ban of a wide range of drugs for animal production (McNulty *et al.*, 2007; Huyghebaert *et al.*, 2011). Prebiotics are non-digestible food ingredients that are selectively fermented by gut bacteria and are known to have positive effects on Gastrointestinal (GI) physiology. Some prebiotics have been shown to selectively stimulate the growth of endogenous lactic acid bacteria in the gut thereby improving the health of the host (Gibson and Roberfroid, 1995). Prebiotics selectively modify the colonic microflora and can potentially influence gut metabolism (Gibson and Wang, 1994). The commercially available mycelium product of *Aspergillus niger*, Fermacto® (PetAg Inc. Hampshire, IL 60140 USA), referred to as *Aspergillus* Meal (AM), has no live cells or spores and is proven to enhance the digestive efficiency of the GI tract (Harms and Miles, 1988; Potter and Shelton, 1984). *Aspergillus* fiber contains beta-glucans (McCleary and McCleary, 2000),

Fructooligosaccharides (FOS) (Sangeetha *et al.*, 2004), chitosan (Jonker *et al.*, 2010; Muzzarelli, 2010) and Mannanooligosaccharides (MOS) (Uchima *et al.*, 2011; Vera *et al.*, 2011). Beta-glucan is considered as a powerful immune-enhancing nutritional supplement that affects the intestinal villi and primes the innate immune system to help the body defend itself against viral and bacterial invaders (Tsukada *et al.*, 2003; Lowry *et al.*, 2005; Jonker *et al.*, 2010). MOS protect the GI tract from invading toxins and pathogens by binding toxin active sites (Biggs *et al.*, 2007). FOS and chitosan refer to a class of non-digestible carbohydrates that are readily fermented by beneficial bacteria in the intestine. A healthy population of these beneficial bacteria in the digestive tract enhances the digestion and absorption of nutrients, detoxification and elimination processes and helps boost the immune system (Chow, 2002; Tokunaga, 2004; No *et al.*, 2007; Kong *et al.*, 2010). With an increase in the dependence on livestock as an important food source, it becomes crucial to achieve good health in order to make rearing of animal food sources safe and beneficial to both animals and humans. The objective of the present study was to evaluate the effect of 0.2% dietary *Aspergillus* meal prebiotic in reducing *Salmonella* colonization and transmission in turkeys and broiler chickens.

Table 1: Ingredient composition and analysis of the experimental diets (percentage, as fed)

Ingredient	Chicken feed	Chicken feed plus <i>Aspergillus</i> meal	Turkey feed	Turkey feed plus <i>Aspergillus</i> meal
Corn	62.30	62.30	43.90	43.90
Soybean meal	30.00	30.00	49.70	49.70
Fat	3.10	3.10	2.30	0.83
Dicalcium phosphate	1.60	1.60	3.20	3.20
Calcium carbonate	1.50	1.50	0.95	0.95
Trace minerals	0.10	0.10	0.20	0.20
Salt	0.40	0.40	0.37	0.37
Choline Chloride	0.20	0.20	0.25	0.25
Vitamin premix ¹	0.20	0.20	0.50	0.50
DL-Methionine	0.21	0.21	0.17	0.17
L-lysine 98%	0.23	0.23	0.25	0.25
L-threonine 98%	0.11	0.11	0.15	0.15
Saycox	0.01	0.01	0.02	0.02
<i>Aspergillus</i> meal ²		0.20		0.20
Calculated analysis				
Crude protein ³ (%)	21.20	21.30	37.90	38.00
ME (Mcal/kg)	3.108	3.108	2.30	2.30
Lysine (%)	1.19	1.19	1.15	1.15
Methionine (%)	0.50	0.50	0.34	0.34
Threonine (%)	0.79	0.79	0.82	0.82
Calcium (%)	0.99	0.99	0.87	0.87
Phosphorus (%)	0.68	0.68	1.50	1.50

¹The vitamin and mineral premix contained vitamins and trace elements to meet the requirements specified by NRC (1994). All the experimental diets were designed to be low in P. The premix provided (units/kg diet): retinol 3600 mg, cholecalciferol 125 mg, -tocopherol 34 mg, menadione 3 mg, thiamine 2 mg, riboflavin 7 mg, pyridoxine 5 mg, cobalamin 15 mg, nicotinic acid 50 mg, pantothenic acid 15 mg, folic acid 1 mg, biotin 200 mg, iron 80 mg, copper 10 mg, manganese 100 mg, cobalt 05 mg, zinc 80 mg, iodine 1 mg, selenium 02 mg and molybdenum 05 mg.

²Added after batch-mixing (on top of the formula).

³Laboratory analyzed (AOAC, 1995)

MATERIALS AND METHODS

Salmonella challenge: Primary poultry isolates of *Salmonella enterica* serovar Enteritidis (SE) and *Salmonella enterica* serovar Typhimurium (ST) resistant to Novobiocin (NO) (Catalog No. N-1628, Sigma, St. Louis, MO 63178) and originally obtained from the National Veterinary Services Laboratory (Ames, Iowa) were used in these experiments. These isolates were selected for resistance to Nalidixic Acid (NA) (Catalog No. N-4382, Sigma, St. Louis, MO 63178). For these experiments, SE and ST were grown in Tryptic Soy Broth (TSB) (Catalog No. 211822, Becton Dickinson, Sparks, MD 21152) for approximately 8 h at 37°C. The cells were washed three times with 0.9% sterile saline by centrifugation (3,000 x g) and the concentration of the stock solution was determined spectrophotometrically. The stock solution was serially diluted and confirmed by colony counts of three replicate samples (0.1 mL/replicate) that were spread plated on Brilliant Green Agar (BGA) (Catalog No. 278820, Becton Dickinson, Sparks, MD 21152) plates containing 25 µg/mL of NO and 20 µg/mL of NA. The colony-forming units (cfu) of *Salmonella*, determined by spread plating, were reported as the concentration of *Salmonella* (in cfu/mL) for the challenge of the experiments.

Diet composition and preparation: For all experiments, birds were fed an unmedicated corn-soybean starter

diet. The feed composition shown in Table 1 met NRC requirements (NRC, 1994). *Aspergillus* meal (PetAg Inc. Hampshire, IL 60140 USA) categorized as generally recognized as safe (AAFCO, 2011) was added at a concentration of 0.2% and labeled as treated feed, while controls were fed non-treated feed.

Experiment 1: All animal care protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Arkansas. Experiment one was conducted to evaluate the effect of dietary 0.2% AM against horizontal transmission of SE in turkeys. One hundred twenty day-old turkeys obtained from a commercial hatchery (Cargill Inc, Gentry, AR) were randomly assigned to two treatment groups-Control (N = 60) and 0.2% AM (N = 60) and placed in floor pens on fresh pine wood shavings. The respective diets and water were provided *ad libitum*. Five additional seeder turkeys per group were identified with a neckband and challenged with 1.5×10^5 cfu SE on the day-of-hatch and placed in each of the treatment groups 24 h later. At ten, twenty and thirty days of age, turkeys were humanely killed by CO₂ inhalation and Cecal Tonsils (CT) were aseptically removed. *Salmonella* recovery procedures have been previously described by our laboratory (Tellez *et al.*, 1993). Briefly, cecal tonsils were enriched in 10 mL of tetrathionate broth (Becton Dickinson, Sparks, MD) overnight at 37°C. Following enrichment, each sample

was streaked for isolation on BGA plates containing 25 µg/mL NO and 20 µg/mL NA. The plates were incubated at 37°C for 24 h and examined for the presence or absence of typical lactose-negative colonies of SE. Seeder birds were not cultured for SE recovery.

Experiments 2 and 3: Two independent trials were conducted to evaluate the effect of dietary 0.2% AM against horizontal transmission of ST in chickens. Forty day-old chickens obtained from a commercial hatchery (Cobb-vantress, Siloam Springs, AR) were randomly assigned to two treatment groups-Control (N = 20) and 0.2% AM (N = 20) and placed in floor pens on fresh pine wood shavings. The respective diets and water were provided *ad libitum*. In each experiment, five additional seeder chicks per group were identified with a neckband and challenged with 1.25×10^5 cfu of ST at hatch and placed in each of the treatment groups 24 h later. At 10 days of age, Liver and Spleen (L/S) as combined samples and CT were collected from contact chicks in each group for ST recovery. Briefly, organs were enriched in 10 mL of tetrathionate broth overnight at 37°C. Following enrichment, each sample was streaked for isolation on BGA plates containing 25 µg/mL NO and 20 µg/mL NA. The plates were incubated at 37°C for 24 h and examined for the presence or absence of the antibiotic resistant ST. Seeder birds were not cultured for ST recovery.

Statistical analysis: Chi-square test of independence, on a Microsoft excel program, was used to compare *Salmonella* recovery between control and treated groups (Zar, 1984). Significant differences were determined at $p < 0.05$.

RESULTS

In experiment 1, a significant reduction of SE cecal tonsil colonization was observed in turkeys that received dietary AM when compared with controls at all points of evaluation ($p < 0.05$). At days 10, 20 and 30, CT of non-treated birds were SE positive at 100%, 90% and 75%, respectively. However, treated birds demonstrated a 25%, 30% and 35% reduction in CT recovery of SE at the same time-points (Table 2). In both experiments 2 and 3, dietary AM significantly reduced liver/ spleen invasion and cecal tonsils colonization of ST when compared with non treated chickens. In experiment 2, at day 10, L/S and CT of non-treated birds were ST positive at 90% and 100%, respectively. However, treated birds demonstrated a 60% and 75% reduction in L/S and CT recovery of ST (Table 3). In experiment 3, at day 10, L/S and CT of non-treated birds were ST positive at 95% and 90%, respectively. However, treated birds demonstrated a 55% and 60% reduction in L/S and CT recovery of ST (Table 3).

DISCUSSION

Several studies have demonstrated that prevention of *Salmonella* colonization in chickens can be achieved by feeding prebiotics (Babu and Raybourne, 2008; Donalson *et al.*, 2008). *Aspergillus* meal prebiotic contains beta-glucans (McCleary and McCleary, 2000), Fructooligosaccharides (FOS) (Sangeetha *et al.*, 2004), chitosan (Jonker *et al.*, 2010; Muzzarelli, 2010) and Mannanooligosaccharides (MOS) (Uchima *et al.*, 2011; Vera *et al.*, 2011) in its composition. Functionally, the innate immune system of immature chicks is inefficient during the first week post-hatch (Kogut and Klasing, 2009). This immunological inefficiency enables

Table 2: Experiment 1, effect of dietary *Aspergillus* meal against horizontal transmission of *Salmonella enterica* serovar Enteritidis at 10, 20 and 30 days of age in turkeys

Groups	Day 10 Cecal Tonsils	Day 20 Cecal Tonsils	Day 30 Cecal Tonsils
Control - No AM	20/20 (100%)	18/20 (90%)	15/20 (75%)
AM	15/20 (75%)*	12/20 (60%)*	8/20 (40%)*

Notes: Five additional seeder turkeys per group were identified with a neckband and challenged with 1.5×10^5 cfu SE on the day-of-hatch and placed in each of the treatment groups 24 h later. At ten, twenty and thirty days of age, turkeys were humanely killed by CO₂ inhalation and Cecal Tonsils (CT) were aseptically removed. Seeder turkeys were not included in the culture. Data expressed as SE positive/total turkeys for each tissue sampled (%). N = 20/group.

*Asterick within columns of experimental groups indicates significant difference at $p < 0.05$. AM = *Aspergillus* Meal; SE = *Salmonella enterica* serovar Enteritidis

Table 3: Experiments 2 and 3, effect of dietary *Aspergillus* meal against horizontal transmission of *Salmonella enterica* serovar Typhimurium at 10 days of age in chickens

Groups	Experiment 2		Experiment 3	
	Liver/Spleen	Cecal Tonsils	Liver/Spleen	Cecal Tonsils
No AM	18/20 (90%)	20/20 (100%)	19/20 (95%)	18/20 (90%)
AM	6/20 (30%)*	5/20 (25%)*	8/20 (40%)*	6/20 (30%)*

Notes: In each experiment, five additional seeder chicks per group were identified with a neckband and challenged with 1.25×10^5 cfu of ST at hatch and placed in each of the treatment groups 24 h later. At 10 days of age, Liver and Spleen (L/S) as combined samples and CT were collected from contact chicks in each group for ST recovery. Seeder chicks were not included in the culture. Data expressed as ST positive/total chickens for each tissue sampled (%). N = 20/group.

*Asterick within columns of experimental groups indicates significant difference at $p < 0.05$. AM = *Aspergillus* Meal; ST = *Salmonella enterica* serovar Typhimurium

pathogens such as *Salmonella* spp. to invade and colonize the visceral organs of immature chicks. Beta-glucan is a non-soluble fiber that may function as an immuno-potentiator in the digestive tract (Tsukada *et al.*, 2003). According to Lowry *et al.* (2005), dietary beta-glucan reduces SE colonization significantly in chickens. In their experiment, SE from L/S was recovered from 76% of non-treated birds, while only 7% of the birds were positive for SE in the treated group. Moreover, in the same study, heterophils isolated from birds treated with dietary beta-glucan contained 40% ($p < 0.05$) more SE than heterophils isolated from untreated birds. Heterophils form the first line of defense while their killing of *Salmonella* is well-described. This corroborates the immunostimulatory effect of beta-glucans.

Fructooligosaccharides are widely used as prebiotics in a broad range of animal species and these carbohydrates have been tested with success for protection against *Salmonella* infections in chickens as shown by Fukata *et al.* (1999). In a series of experiments, 0.1% FOS was able to reduce SE colonization in chickens significantly. Also, MOS as a prebiotic has been shown to reduce the prevalence and concentration of *Salmonella*, *E. coli* and *Clostridia* in the intestinal tract of chickens (Babu and Raybourne, 2008; Donalson *et al.*, 2008; Fukata *et al.*, 1999; Van Immerseel *et al.*, 2004; Lenoir-Wijnkoop *et al.*, 2007; Kim *et al.*, 2011). Kim *et al.* (2011) conducted a study where dietary MOS (0.05%) and FOS (0.25%) had an effect on intestinal microflora of broiler chickens, suggesting the use of these prebiotics as an alternative to the use of growth-promoting antibiotics. Finally, chitosan is a modified, natural biopolymer derived by deacetylation of chitin, the main component of the cell walls of fungi and exoskeletons of arthropods. Chitosan exhibits numerous beneficial effects, including strong anti-microbial and anti-oxidative activities (No *et al.*, 2002; Friedman and Juneja, 2010). Its application in agriculture, horticulture, environmental science, industry, microbiology and medicine are well reported (No *et al.*, 2002). According to Huang *et al.* (2005), the use of 0.01% or 0.015% of oligochitosan in the diet increased serum levels of immunoglobulins in broiler chickens, suggesting a potential immunomodulatory effect. There have been numerous studies that report the use of chitosan as a mucosal adjuvant, by enhancing IgA levels. It is well known that IgA is active across mucosal surfaces and is the predominant class of antibody against enteric pathogens (Huang *et al.*, 2005; Lubben *et al.*, 2001; Rauw *et al.*, 2010). The commercial prebiotic supplement derived from *Aspergillus* sp. mycelium is unique because it contains all of the above mentioned prebiotic ingredients. Additionally, AM contains 16% protein and 45% fiber (Harms and Miles,

1988) and may be used with low levels of protein and amino acid diets to improve performance in commercial poultry (Torres-Rodriguez *et al.*, 2005). Even though the exact mechanisms of action for prebiotics have not been defined, it may be speculated that the effect is due to changing intestinal flora that promotes the growth of beneficial bacteria. This product has also been shown to benefit poultry through stimulation of growth, most probably by increasing absorption of feed ingredients and improving digestibility (Potter and Shelton, 1984; Harms and Miles, 1988). Nevertheless, this product was never tested for its effect against pathogenic microorganisms, especially *Salmonella* sp., which are known to profusely colonize poultry. The reduction of *Salmonella* levels in poultry is critical to prevent transmission to human populations. Therefore, control mechanisms are aimed at increasing the overall food safety in addition to better welfare of poultry (Ljungh and Wadstrom, 2006; Foley *et al.*, 2011). The results of this study showed that dietary supplementation with 0.2% *Aspergillus* meal was able to reduce *Salmonella* Enteritidis horizontal transmission in turkeys and *Salmonella* Typhimurium horizontal transmission in broiler chickens, by reducing the overall colonization levels in birds. Although the mechanism of action is not totally understood, the reduction in *Salmonella* colonization may be related to a synergistic effect between beta-glucan, MOS, chitosan and FOS present in the *Aspergillus niger* mycelium. More studies are needed to delineate the exact roles played by these components and future studies will also test this prebiotic in combination with probiotics to see whether a symbiotic effect can be established.

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