COMPARISON OF PROPHYLACTIC OR THERAPEUTIC DIETARY ADMINISTRATION OF CAPSAICIN OLEORESIN FOR RESISTANCE TO SALMONELLA IN BROILER CHICKENS

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Comparison of prophylactic or therapeutic dietary administration of capsaicin oleoresin for resistance to *Salmonella* in broiler chickens

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ABSTRACT

Expt. 1 evaluated effects of 0 or 10 ppm CAP in the starter phase (d 1-16) on chicks challenged with SE on d of age. Therapeutic inclusion of 10ppm CAP increased ($P < 0.05$) L/S and ceca positives. In Expt. 2, capsaicin oleoresin (CO) was included in finisher diets (d 30-37) at 0, 5, or 20 ppm with SE challenge on day 31. Inclusion of 5 ppm CO increased ($P < 0.05$) ceca SE positives and demonstrated 1.05 and 1.39-log fewer SE cfu at CO concentration of 5 or 20 ppm, respectively. A linear decrease ($P < 0.05$) in lamina propria thickness of SE challenged birds was observed with increased CO. Expt. 3 evaluated prophylactic CO treatment at 0, 5, or 20 ppm in starter, grower, and finisher diets for resistance to SE or ST challenge on d 14 or 29. With challenge on d 14, 5 ppm CO reduced ceca ($P<0.005$) SE positives and 1.1-log fewer SE cfu. Likewise, 20 ppm CO reduced ($P < 0.05$) SE ceca positives. *Salmonella typhimurium* isolation rate was reduced ($P<0.05$) with 5 ppm CO, and ST cfu were reduced 1.4-log with 5 ppm CO compared to 20 ppm. Lamina propria thickness increased ($P < 0.05$) linearly as CO concentration increased. With d 29 challenge birds fed 5 ppm CO exhibited 1.08-log fewer SE cfu, and 20 ppm CO reduced L/S positives ($P < 0.025$) for SE and resulted in 1.39-log fewer SE cfu. Lamina propria thickness decreased with 5 ppm CO and SE or ST challenge compared to non-challenged birds fed 5 ppm ($P < 0.0005$). An increase was observed in ST or SE, birds fed 20 ppm CO compared to non-challenged, birds fed 20 ppm CO ($P < 0.01$). No differences were observed in mast cell number in either Expt. 2 or 3.

<p>These data provide evidence that prophylactic or therapeutic dietary CAP differentially affect broiler susceptibility to *Salmonella* and prophylactic administration may provide non-antibiotic means to reduce *Salmonella* in broilers.

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INTRODUCTION

Salmonella is a major cause of food-borne illnesses in the US. Many cases of human salmonellosis can be attributed to the presence of *Salmonella* on poultry and poultry products. Numerous serotypes of *Salmonella* are showing evidence of resistance thereby increasing the risk to humans who are potentially consuming these organisms found on meat and egg products. The increased prevalence of antibiotic resistant microorganisms is compounded due to the prophylactic use of antibiotics in animal feed. Due to these concerns, the commercial poultry industry has had an increased interest in finding non-antibiotic means to reduce the presence of *Salmonella* organisms both on the farm and on carcasses in processing plants. Capsaicin (CAP), the pungent component of peppers, has been reported to decrease the presence of *Salmonella enteritidis* (SE) in the organs of broiler and leghorn chickens when administered in the diet (Tellez, et al., 1993; McElroy et al., 1994; Tellez et al., 1994). Research currently aims to elucidate the mechanism by which this decrease occurs. Our laboratory is particularly interested in the mechanism of action and host response to CAP in correlation with immuno-modulatory and morphological effects. The purpose of the present studies was to evaluate the effects of prophylactic or therapeutic inclusion of CAP, in purified or capsaicin oleoresin (CO) form, in the diet of broilers at different points during growout for resistance to *Salmonella*. We hypothesized that CO provides a non-antibiotic means to reduce *Salmonella* in broilers when administered in the diet prior to or post-challenge. Also, that variability does exist between duration of CAP administration and subsequent challenge with *Salmonella,* and that this is attributed to differences in lamina propria thickness and mast cell numbers in small intestinal lamina propria. *Salmonella enteritidis* and *Salmonella typhimurium* (ST) were used in these studies due to the high prevalence of these serotypes isolated from cases of human salmonellosis.

REVIEW OF LITERATURE

Salmonella

Salmonella is a genus of rod-shaped, gram-negative, facultative anaerobic bacteria of the family Enterobacteriaceae. They are usually motile with peritrichous flagella and are non-spore forming. The flagella determine the serovar type and are necessary for the colonization of host tissue. Currently, more than 2300 serotypes of *Salmonella* are known to exist (White et al., 1997). *Salmonella* have many different virulence factors that aid in their pathogenicity including: adhesion (through Fim I fimbriae involving receptors on the epithelium), invasion, production of entero- and cytotoxins, lipopolysaccharide (LPS), intracellular survival and multiplication, and virulence plasmids (Suzuki, 1994; Lillehoj and Okamura, 2003).

One of the most common modes of infection is via the fecal-oral route. Once ingested, the *Salmonella* bacterium proceed through the alimentary tract, where they are able to adhere to and invade the intestinal epithelial cells (Suzuki, 1994). This adhesion is mainly observed in epithelial cells near the cecal lumen (Desmidt et al., 1998), and it is likely that the uptake of bacteria occurs in mature enterocytes at the villus tips (Amin et al., 1994). Evidence suggests that ST exhibits a cellular invasion in pigs as early as 10 minutes post-inoculation (Meyerholz and Stabel, 2003). After penetrating the intestinal wall, some *Salmonella* can then invade, survive, and multiply in the reticuloendothelial system (ie: membrane-bound vacuoles of cells in the lamina propria) (Barrow et al., 1987; Desmidt et al., 1998).

Enterotoxins have been identified in a number of genera of enteric bacilli. Jiwa (1981) showed that of 68 *Salmonella* isolates representing 39 serotypes, including *S. enteritidis*, 66 of the isolates tested positive for production of a heat-labile enterotoxin. This enterotoxin affects many cells by altering their function. Cytotoxins also have been present as a result of *Salmonella* infection. They are responsible for inhibition of protein synthesis in intestinal epithelial cells (Koo and Peterson, 1982; Koo et al., 1984), which probably accounts for morphological alterations of the intestinal mucosa (Suzuki, 1994).

Cell wall lipopolysaccharides (LPS) aid in *Salmonella* virulence and consist of three components: lipid A, inner core oligosaccharide, and O-side chain oligosaccharide.

The presence of LPS and different virulent factors appear to contribute to activation of the complement cascade of the immune response. However, one of the most important contributions of LPS to bacterial virulence is that it enables the organism to resist macrophage phagocytosis and colonize the invaded tissues (Suzuki, 1994). These virulence factors are often host-specific allowing some hosts to serve as carriers while others experience disease associated with the microorganism.

Salmonella: a food-borne disease

In the U.S., approximately five million cases of food-borne illness occur each year (White et al., 1997). Salmonellosis accounts for nearly half of these cases. S*almonella typhimurium* (ST) and *Salmonella enteritidis* (SE) are responsible for the majority of food-borne enteritis caused by *Salmonella* in humans (White et al., 1997; Lillehoj and Okamura, 2003). Symptoms of infection include mild fever, nausea, chills, abdominal pain and diarrhea; septicemia and in rare cases death. The incubation period is generally between 12 and 36 hours (Madié and Peterson, 1992). Since salmonellosis is considered one of the three most common food-borne diseases in the western world, *Salmonella* has become a microorganism of interest in the public health field.

Industry Concerns

Due to the many different virulence factors, identifying the specific mechanism of *Salmonella* invasion and persistence in different species is difficult. Generally *Salmonella* are commensal in chickens, which usually can carry the organism with little or no symptoms (White et al., 1997). *Salmonella* and *Campylobacter* are considered to carry the highest risk for food-borne illnesses in comparison to other microorganisms linked to poultry (White et al., 1997). Poultry meat and eggs are the major sources of salmonellosis in humans, and poultry are regarded as the largest natural reservoir of *Salmonella* organisms (Madié and Peterson, 1992). Since poultry are implicated as one of the major causes of human salmonellosis, the industry has applied risk reduction strategies both on-farm and in the processing plants to reduce food-borne diseases. These strategies include: sanitation, biosecurity, vaccine and drug therapy, biological control procedures such as those aimed at preventing colonization, and those implemented at critical control points in the processing plant to reduce cross contamination of *Salmonella*- negative chickens in the plant (White et al., 1997). Despite these efforts, SE

and ST are still present in poultry, and controlling them is difficult primarily due to an inadequate understanding of the mechanisms that allow *Salmonella* to persist in poultry (Lillehoj and Okamura, 2003).

Direct contact (horizontal transmission) with clinically ill or symptomless birds, or consumption of contaminated feed or water are the main routes by which poultry become infected with *Salmonella* (White et al., 1997). It is also evident that hen to chick transmission (vertical transmission) of SE and ST occurs at the hatchery (Heyndrickx et al., 2002). The primary site of SE infection in young chicks is the ceca where the organism invades the mucosa (Tellez et al., 1994).

In a recent study, ten out of eighteen broiler flocks from different farms sampled on the farm (cecal droppings and plastic boots) were found to be *Salmonella* positive (Heyndrickx et al., 2002). Of the positive flocks, only six were positive after 42 days of rearing demonstrating the ability of the chicken to generate an immune response and resist invasion. It was evident that antibiotics used at subtherapeutic or therapeutic levels during rearing aided in decreasing infection in these flocks; however, overall, 17 of the 18 flocks had carcasses leaving processing that were *Salmonella* positive (Heyndrickx et al., 2002). Other studies show similar results reporting only 10% of chickens entering processing as *Salmonella* positive, while that number increased to 30-50% of carcasses positive for *Salmonella* before and after defeathering (Waldroup, 1996). Evidence that stress of transport can increase shedding of the microorganism and that cross contamination can occur at processing (Heyndrickx et al., 2002), could mean that the pathogen load in poultry that arrive for slaughter may affect the presence of the microorganism on the processed carcasses (White et al., 1997).

Currently, the poultry industry doesn't guarantee a *Salmonella*-free product; however, when the USDA/Food Safety and Inspection Service (FSIS) focused on establishing a microbiological baseline for broiler chickens, *Salmonella* was recovered from 20% of the broiler carcasses analyzed. Of these positive carcasses, more than 95% of them yielded three or fewer *Salmonella* organisms per cm^2 making these carcasses a low health risk if prepared using ordinary cooling and cooking practices (White et al., 1997). Consumers however, demand higher quality standards for their food, and due to

the high costs associated with salmonellosis, decreasing the contamination of carcasses would be advantageous.

It has been estimated that the cost of infectious intestinal diseases in the United States was $23 billion in 1985 (Garthright et al., 1988). Food-borne bacterial diseases have been estimated at $4.8 billion for 1987 with salmonellosis accounting for $1.4 billion of the total (Roberts, 1988). More recently, the economic impact of food-borne pathogens has been estimated at $6 billion (Lillehoj and Okamura, 2003). Consequently, the economic burden of this disease to both the consumer and the poultry industry is great. With increasing pressure to decrease the prevalence of *Salmonella*, it is necessary for investigators to clearly elucidate *Salmonella* pathogenicity in chickens.

Host Pathogenicity

The immune response of chickens to *Salmonella* involves both innate and acquired immunity (Lillehoj and Okamura, 2003). Gastrointestinal immunity can be observed both on a localized and systemic level. The gastrointestinal epithelium is covered by a mucus layer made of mucin glycoproteins that are synthesized and secreted by goblet cells. This layer can provide a barrier against invasion of microbes (Albanese et al., 1994). Other non-specific barriers to microbial invasion are gastric secretions, lysozymes, bile salts, microbial flora, and endogenous cationic peptides (Lillehoj and Okamura, 2003). It is known that invasion of *Salmonella* causes an inflammatory response characterized by the arrival of large numbers of heterophils and mononuclear leukocytes (Porter and Holt, 1993). When leghorn chicks were exposed to SE of various phage types (4, 8, and 5A) at one day of age, mortality only occurred in a few groups, however, there were no significant differences between challenged groups as compared to control. Histopathology obtained from dead birds (15.3% challenged with phage type 5A; 7.6% challenged with phage type 4-chicken-CA) showed severe yolk sac infection, peritonitis, and enteritis. Overall however, the chickens were able to overcome the infection and did not show many clinical signs (Dhillon et al., 2001).

Changes in the lymphocyte population could provide insight as to how chickens are responding to *Salmonella* invasion. B and T lymphocyte populations change as early as one day after exposure to SE. Humoral antibodies (IgA, IgG, and IgM) from serum and intestinal mucosal samples of chickens challenged with ST significantly increased

one week post-challenge (Brito et al., 1993). Sasai et al. (2000) found that IgG^+ B lymphocytes in the cecal tonsils of challenged leghorns were significantly higher at 6 days post-inoculation than in control groups. Also, $CD4^+$ T lymphocytes were increased at 4 days post-inoculation in groups challenged with $1x10^9$ CFU as compared to those challenged with $1x10^6$ CFU and control groups (Sasai et al., 2000). SE infection has been shown to increase IgA, IgM, and IgG production in the reproductive organs of hens (Withanage et al., 1998; Withanage et al., 1999). Once *Salmonella* invade the system, usually through capture and transport within host macrophages, antibodies may not provide adequate protection against infection. They may aid in mucosal immunity however, through the macrophage-mediated opsonization and destruction of the bacteria (Lillehoj and Okamura, 2003).

Morphological changes in the intestine of birds have also been observed in response to *Salmonella* infection. Degeneration of the intestinal mucosal cells were observed three weeks post-challenge with sporadic disappearance of surface microvilli in male day-old chicks challenged with ST (Bayer et al., 1977). Additional morphological changes observed in other hosts have not yet been documented in chickens. Investigators have found that in mice, ST invasion limits the uptake of amino acids (L-alanine, L-lysine, and L-aspartate) and subsequent synthesis of enterocyte proteins, and like in chickens, is associated with neutrophil infiltration (Mehta et al., 1998). Along with infiltration of neutrophils, morphologic differences observed in calves, such as blunting of absorptive villi, inflammatory changes and detachment of epithelial cells from the villus tip have been found as early as 15 minutes, 1 hour, and 3 hours post-intralumenal infection with ST, respectively (Santos et al., 2002).

Antibiotic Resistance

Salmonella typhimurium DT104 is resistant to many antibiotics. Carlson et al. (2001) found that ST DT104 possessed a phenotype giving the microbe cytotoxic capabilities that caused abomastitis in veal calves and increased pathogenicity. According to a report in 2002 from the Center for Disease Control, 53% of ST isolates were resistant to one or more drugs and 30% had a five-drug resistant pattern. Many drug resistant microorganisms are posing a serious health threat to hospital patients. More than 70% of the bacteria found to cause hospital-acquired infections are resistant to

at least one of the drugs commonly used to treat them (NIAID, 2004). As the number of drug resistant microorganisms increases, the need to find other means to control and treat illness is more immediate.

Capsaicin

Capsaicin (8-methyl-N-vanillyl-6-nonenamide) is the pungent constituent of a variety of fruits from the genus capsicum, such as hot peppers (Surh and Lee, 1995; Yu et al., 1998). It is used as a food additive throughout the world, particularly in South East Asian and Latin American countries. It is currently being utilized for therapeutic treatment of various peripheral conditions such as rheumatoid arthritis and diabetic neuropathy (Surh and Lee, 1995) and is described as a neurotoxin and tachykinin-releaser (Powell et al., 1991). Capsaicin is a relatively lipophilic molecule and affects the fluidity and/or ion permeability of the plasma membrane allowing Ca^{2+} and possibly other cations to move across the membrane (Govindarajan and Sathyanarayana, 1991). Hammer et al. (1998), however, found no significant changes in water and electrolyte absorption in human jejunum with three different concentrations of CAP (5, 10 or 50 μg/ml).

The action of CAP has been studied in mammalian species. Nociceptors can be excited by different stimuli and can trigger the release peptides and neurotransmitters (ie: substance P and calcitonin-gene-related peptide). They also have thresholds or sensitivities that are uncommon to other sensory nerve fibers (Julius and Basbaum, 2001). Upon ingestion of CAP, a tingling and burning pain is activated by VR1, a nonselective cation channel located on C fiber primary sensory nerve endings. These receptors possess a steep temperature dependence and a thermal activation threshold of about 43C (Caterina et al., 1997; Julius and Basbaum, 2001). Birds exhibit sensation of heat like mammalian species, however their primary sensory neurons are insensitive to CAP. Due to this, birds may express a vanilloid-insensitive homolog of VR1 (Jordt and Julius, 2002) rendering them insensitive to the irritant effects of CAP (Tellez et al., 1993; Jordt and Julius, 2002). Tellez et al. (1993), and McElroy et al. (1994) demonstrated that the presence of CAP in the feed of leghorn or broiler chickens did not cause any changes in feed intake, or significant differences in body weight or feed efficiency. The receptor possessed by chickens is however, activated by heat or protons (Jordt and Julius, 2002). Mice lacking VR1 showed normal behavioral responses to temperatures near the

threshold for VR1 activation. However, they exhibited reduced pain behavior at higher temperatures demonstrating their inability to discriminate among stimuli of different intensities (Julius and Basbaum, 2001).

It has been proposed that CAP is metabolized by hepatic microsomal mixed-function oxidases. This mechanism can exert harmful effects such as necrosis, mutagenesis, and carcinogenesis (Surh and Lee, 1995). Current research on the actual localized and systemic effects of CAP remain inconclusive. One study reviewed by Surh and Lee (1995) stated that CAP was found to be mutagenic in strains of ST (TA98, TA100, and TA1535) tested in mice resulting in antibiotic resistance. Another study, however, found that neither commercial capsaicinoids nor those isolated from chili extracts were mutagenic to ST (Vinitketkumnuen et al., 1991). In Swiss albino mice fed dietary CAP at 0.5, 0.25, 0.125, or 0.0625%, duodenal tumors were found (Toth et al., 1984), but none were apparent in groups receiving the highest concentration of CAP (1%) (Toth et al.,1984; Toth and Gannett, 1992). This could illustrate that the effects of CAP are dose dependent. Also, in both of these studies, evidence showed that females were possibly more affected by CAP than males who developed fewer tumors than females. A study conducted in Mexico found that consumption of chili pepper increased the risk of developing gastric cancer (Lopez-Carillo et al., 1994). Park et al. (1998), however, indicated that repeated administration of CAP on the backs of mice led to no significant increase of papilloma on the skin.

Capsaicin has also been shown to enhance immune responses. Nilsson et al., (1990) reported a close association of mast cells to peptidergic nerves, which affects inflammatory conditions. No effect was seen on the phagocytic activity of peritoneal macrophages with 20ppm dietary CAP administration in mice, however, an increase in the level of tumor necrosis factor α (TNFα) was observed (Yu et al., 1998) possibly resulting in an increased influx of chemotactic cells aiding the mucosal immune response. Evaluating the effect of CAP and substance P on eosinophilic granule cells (EGCs), Powell et al. (1991) found that degranulation of EGCs was observed in the lamina propria and stratum compactum of the peritoneum. This degranulation was reminiscent of mast cell degranulation and endocrine cell crinophagy. Total degranulation was evident with the presence of extracellular granular material. Degranulation it appears, is initiated and

mediated by substances (probably tachykinins) released from the closely associated non-myelinated nerves found in the lamina propria (Powell et al., 1991).

Lymphocytes are also affected by presence of CAP. Cecal tonsils contain primarily IgG^+ and IgM^+ bearing B cells (Sasai et al., 2000). In mice fed 20 or 50ppm CAP, total serum levels of both IgG and IgM were significantly increased. The maximum productions of these immunoglobulins were in mice receiving 20ppm CAP, and proliferation of T lymphocytes was significantly increased in response to Con A (T-cell mitogen) in mice that received 20ppm CAP as compared to controls (Yu et al., 1998). In contrast, in rats treated with 50mg/kg CAP, subcutaneously, the antibody response was decreased more than 85% (Helme et al., 1987). Also, the ability of rats pretreated with 50mg/kg CAP, subcutaneously, to respond to sheep red blood cells was decreased due to a deficiency in IgM secretion (Eglezos et al., 1990).

Capsaicin has been found to increase the secretion of immunoreactive neuropeptides such as catecholamines (Holzer et al., 1980), neurokinin A, vasoactive intestinal polypeptide, calcitonin gene-related peptide (Rawdon, 1984; Holzer, 1990), and substance P (SP) (Surh and Lee, 1996). Neurotransmitters have been implicated in mediating the release of vasoactive compounds from mammalian mast cells. Substance P is a neuropeptide found in the brain and intestinal tissue that modulates the inflammatory reaction and immuno-responsiveness of the host (Helme et al., 1987; Tellez et al., 1993) causing vasodilation and increasing contraction of gastrointestinal smooth muscle. Substance P has also been shown to induce histamine release from mast cells (Devillier et al., 1985). Systemic injection of CAP is thought to decrease SP in the system (Yu et al., 1998) however, dietary CAP may have different effects. Substance P levels in the spinal cord were reduced 55% in mice injected with 30μg CAP intrathecally. Substance P was only released from unmyelinated primary afferent neurons terminating in the spinal cord and not from other tissues or the hypothalamus. It was reported that the action of CAP on SP is restricted to the primary sensory neurons (Govindarajan and Sathyanarayana, 1991). Capsaicin can destroy the unmyelinated afferent nerves and deplete the peripheral tissues of SP (Helme et al., 1987). This evidence demonstrates that while injected CAP has detrimental effects to immune responses through decreased SP levels, dietary CAP may enhance the immune response.

Bactericidal Capabilities

In leghorn chickens given dietary CAP (18ppm) for 14 or 19 days and orally challenged with SE, cecal colonization was not reduced even though there was a significant decrease in *Salmonella* organ-positive samples. This effect suggests that CAP is not bactericidal (Tellez et al., 1993). Dorantes et al. (2000) investigated three different chili peppers and their possible antibacterial effects in vitro on four bacteria, including ST. Capsaicin and dihydrocapsaicin from habanero, serrano, and morrón peppers did not show any inhibitory effect on bacterial growth. Tellez et al., 1993 found that dietary CAP decreased the pH of the ceca. Suboptimal conditions can induce bacteriostasis in microorganisms. Since no bactericidal effect has been observed with CAP, the immune effects observed may be due to induction of bacteriostatic organisms, or an interaction of *Salmonella* with the host.

CAP-Salmonella Host Interactions

Since many of the immuno-modulatory effects of CAP have been determined in mammals, investigators wanted to elucidate how this pungent compound affects birds, namely chickens. Birds have long been recognized as vectors of pepper seed dispersal and they possess a vanilloid receptor that fails to be activated by CAP (Jordt and Julius, 2002). Histological evidence of a mild to moderate infiltration of mononuclear cells and heterophils in the lamina propria of the ceca was observed with SE inoculation of leghorn chickens fed 18ppm CAP for 14 or 19 days (Tellez et al., 1993). An increase in heterophils, villus height and crypt depth were also observed by Manning et al. (1994) in the presence of 5ppm or 20ppm CAP or SE challenge. Tellez et al. (1993) also found that dietary CAP lowered the pH of the cecal contents, and increased proliferation of epithelial cells. These morphological changes suggest that CAP may alter host susceptibility to SE invasion by allowing decreased microbial proliferation and possible attachment to epithelial cells. McElroy et al. (1994) investigated the effects of dietary CAP on broiler chickens and found a decrease in incidence of SE positive organs and a decrease in cecal tonsil colonization in SE challenged groups fed CAP (5ppm or 20ppm) for 21, 28 or 42 days prior to challenge as compared to controls. The day of challenge did not seem to change the effect of CAP on SE invasion. As a result of *Salmonella*

invasion, an immune response is elicited with evidence of inflammation possibly due to involvement of immune effector cells, such as mast cells.

Mast Cells

Mast cells are strategically located at the host-environment interface and are one of the first cells to come into contact with invading pathogens. They are located in all layers of the gastrointestinal tract, but are mostly found in the lamina propria. They are able to regulate the properties of the epithelial layer that effect transport of molecules through the release of pre-synthesized (histamine, 5-hydroxytryptamine, and tryptase) or newly synthesized (prostaglandins, leukotrienes, platelet-activating factor, TNF-α, IL1-6, IL-8 and IL-18) mediators (Yu and Perdue, 2001). They are a potential source of early response cytokines such as TNF-α and IL-4 that are instrumental in initiating the immune and inflammatory responses of the host to invading pathogens (Feger et al., 2002). Mast cell involvement in immune responses associated with Th2 cells and IgE hypersensitivity responses have been well established (Galli et al., 1999). However, little is known about their involvement in innate immune responses. As stated earlier, Fim I fimbriae adhere to cells allowing *Salmonella* to invade the host. The radial arrangement of these fimbriae probably ensures that FimH is the first bacterial component sterically able to mediate productive interactions with mast cells and neutrophils (Malaviya et al., 1996). This ability of *Salmonella* to adhere to the host as well as the interactions between bacteria and mast cells are important to understand what is necessary for the host to resist infection.

A deficiency of mast cells in the host system has been found to reduce abilities to elicit an adequate immune response. Mast cell-deficient mice were impaired in their ability to kill *Klebsiella pneumoniae* and demonstrated reduced neutrophil infiltration in the lungs, probably a result of lower levels of the mast cell-derived chemotactic TNF-α (Malaviya et al., 1996). Also, mast cell-deficient mice given reconstituted mast cells were able to survive cecal ligation and puncture, however, this effect was reversed when antibodies to TNF-α were administered to the mice (Echtenacher et al., 1996). Cytokines such as TNF-α are released through degranulation, stimulating the action of pro-inflammatory mediators and resulting in both inflammation and edema. Chemotactic factors released from mast cells include histamine, neutrophil chemotactic factor, and eosinophil chemotactic factor (Metcalfe, 1984). Mast cells are long-lived cells (surviving

in some cases for years) and have the ability to respond repeatedly to the same stimulus. These properties enhance their ability to be an effective initial defense against invading pathogens (Feger et al., 2002).

In human and mouse intestinal epithelial lines and in mouse ligated intestinal loops, stem cell factor (SCF) (sometimes referred to as mast cell growth factor) production is enhanced by ST. Stem cell factor:c-kit (SCF receptors) interactions induce a cellular state that doesn't allow bacteria to adequately invade epithelial cells. Pretreating human or mouse intestinal cell lines with SCF results in a cellular state that is resistant to *Salmonella* invasion (Klimpel et al., 1996).

Salmonella is known to survive *in vivo* through expression of toxins that either inhibit the release of inflammatory mediators or lead to their exaggerated release, with severe cytotoxic effects in the surrounding tissues. In opsonin-independent conditions, certain bacteria express adhesins that subvert the phagocytic mechanism of mast cells. This may mean that these cells could serve as reservoirs of viable bacteria in the host (Feger et al., 2002).

Macrophages in the lumen, epithelial lining, and lamina propria have been found to contain the SE organism, demonstrating that they may be involved in clearing the microbe from the tissues (Popiel and Turnbull, 1985). Sher et al. (1979) investigated the mechanisms of mast cell-mediated phagocytosis of bacteria. They reported that mast cells bind to and phagocytose ST and that this interaction is mediated by a complement-derived opsonin. They also observed that ST coated with the iC3b fragment of complement is recognized through complement receptor 3 on the mast cell membrane. After mast cells bind to opsonized bacteria they phagocytize them (Feger et al., 2002). Traditional phagocytes, such as neutrophils and macrophages, can kill bacteria through a combination of oxidative and nonoxidative killing systems, both of which are present in mast cells. There is also evidence for an oxidative bactericidal system in mast cells, which is apparent in the oxidative burst generated by mast cells upon exposure to gram-negative bacteria in opsonizing conditions (Malaviya et al., 1994).

COMPARISON OF PROPHYLACTIC OR THERAPEUTIC DIETARY ADMINISTRATION OF CAPSAICIN OLEORESIN FOR RESISTANCE TO SALMONELLA IN BROILERS CHICKENS

INTRODUCTION

Salmonella live in the environment and can invade many hosts including mammals, birds, and reptiles. *Salmonella* species may be most recognized by the general public due to the fact that they can cause food-borne illness. *Salmonella typhimurium* (ST) is the second most prevalent food-borne disease of bacterial origin in the US (Mead et al., 1999), and ST and *Salmonella enteritidis* (SE) are recognized as the first and second most common serotypes found in humans (CDC, 2002). *Salmonella* is a major contributor to food-borne illnesses linked to poultry products and can be found both in eggs and on poultry carcasses. In humans, salmonellosis causes fever, abdominal pain, and diarrhea with death occurring in rare cases (Madié and Peterson, 1992). Chickens however, can serve as a host to many strains of this organism with little or no symptoms (White et al., 1997). While this can decrease the costs attributed to treatment and mortality, flocks can become *Salmonella* positive and carry the organism into processing plants (Heyndrickx, 2002). Due to this, one focus of the poultry industry is to prevent cross-contamination at critical points in the plant decreasing the risk to consumers. Other methods used to reduce the presence of the organism include drug therapy and vaccination (White et al., 1997). While these methods are effective, they are costly, and this organism remains a major concern.

Currently, there are many concerns with regard to antibiotic use in the poultry industry. It has been estimated that 50% of antimicrobial production has been directed to food-producing animals, and the use of prophylactic drugs may also contribute to the development of drug resistance (IOM, 2004). Many microorganisms can mutate to increase their resistance to antibiotics, and this may pose a potential health threat to humans who consume them on improperly prepared or handled meat. Reducing the presence of *Salmonella* through non-antibiotic means would be advantageous to the industry, allowing, production of a safer product.

Capsaicin (CAP) has been of interest to investigators as a potential non-antibiotic means to reduce *Salmonella* colonization and invasion in chickens. Capsaicin is the pungent component of plants from the genus *Capsicum* (such as bell peppers, jalapenos and habaneros). When administered continuously in the diets of broiler chickens prior to challenge with SE, CAP was found to decrease the SE isolation rate for liver/spleen (L/S)

and cecal tonsils as compared to untreated control (0ppm CAP) groups (McElroy et al., 1994). Dietary CAP (20ppm) has also been shown to affect immune function in mice by enhancing the production of immunoglobulins (Yu et al., 1998). However, birds do not have vanilloid receptors (VR1) for CAP, but may have a homolog VR1 (Jordt and Julius, 2002) resulting in decreased sensitivity to the pungency of this compound.

Our laboratory is currently investigating the effects of CAP on the immune response to *Salmonella* in broilers. Since past research showed a decrease in cecal and L/S isolations in response to CAP (McElroy et al., 1994), it was postulated that intestinal alterations occurred in the ceca, thereby decreasing *Salmonella* colonization. Also, Tellez et al. (1993) observed increases in lamina propria thickness and epithelial cell proliferation with dietary CAP inclusion for 14 or 19 days. Such changes could occur as a result of an influx of effector immune cells as part of an inflammatory response. Nociceptive sensory neurons are sensitive to CAP, and stimulation of these neurons stimulates the release of neuropeptides such as SP (Caterina and Julius, 2001). Substance P is thought to stimulate the release of histamine (Devillier et al., 1985) and induce mast cell degranulation (Shanahan, et al., 1985). Mammalian research has extended the classical role of mast cells in hypersensitivity reactions to include key effector functions in defense to bacterial pathogens (Malaviya et al., 1996; Galli et al., 1999; Feger et al., 2002). To aid in understanding the mechanism of dietary CAP in resistance to SE or ST in chickens, morphological and histological changes in the small intestine were examined in the present experiments. The effect of dose and timing of CAP administration (prophylactic or therapeutic to *Salmonella* challenge) during the growout of broilers was also investigated. The purpose of the present studies was to examine the effects of CAP on host-resistance to SE or ST in correlation with alterations in morphology or mucosal mast cell number. The CAP used in two of these studies (Expt. 2 and Expt. 3) was a spice grade capsaicin oleoresin (CO) (1.08% capsaicinoids), which is less expensive and more conducive to commercial application than the purified CAP used in Expt. 1 and previous investigations (Tellez et al., 1993; McElroy et al., 1994). This oleoresin could be more practically applied in the industry due to availability, economic feasibility, and ease of inclusion in feed. We hypothesized that CO provides a non-antibiotic mean to reduce *Salmonella* infection in commercial broilers when administered in the diet prior to

or post-challenge, and that variability exists between the time of feeding in relation to *Salmonella* challenge and duration of dietary CAP concentration for alterations in mucosal immunity and subsequent resistance of broilers to SE or ST.

MATERIALS AND METHODS

Experimental Animals

In all experiments, straight-run CobbxCobb broilers were obtained from a local commercial hatchery on day-of-hatch. They were placed in floor pens on clean pine shavings at a stocking density of 0.7 ft^2/bird. Ambient temperature was set at 33°C for brooding and decreased to 28°C by day 14 based on bird comfort. Birds were given *ad libitum* access to water and feed. In all experiments negative pressure ventilation was used, and birds were kept under continuous lighting.

Feed

Feed was medicated with a coccidiostat (Avatec) in the starter phase only for Expt. 3, in the starter and grower phases for Expt. 2, and was non-medicated for Expt 1. All diets were formulated at Virginia Tech to meet NRC requirements (1994). For Expt. 1, purified CAP used in Expt. 1 (#M-3403, 60% Capsaicin, Sigma-Aldrich Chemical Co., St. Louis, MO) was obtained in powder form and mixed into the feed after it had been processed. Capsaicin oleoresin (1.08% capsaicinoids, Snyder Seed Co., Buffalo, NY) used in Expt. 2 and 3 was supplied in liquid form and mixed in with the fat source prior to pelleting.

Salmonella

Naladixic acid (NA) and novobiocin (NO) resistant *Salmonella*, SE (phage type 13A) and ST, were used in one or more of the experiments conducted. Stock solutions of *Salmonella* were stored at –80°C, and 10µl of stock were suspended in 20ml tryptic soy broth (TSB) and incubated at 40°C for 8h to permit attainment of log phase. After 8h, a 10µl aliquot was transferred to fresh TSB to allow for a second and third round of growth. Following the last round, cells were washed and centrifuged (2400xg, 4C) three times in phosphate buffered saline (PBS). Washed bacteria were spectrophotometrically quantitated to $1x10^9$ CFU/ml in PBS. Inocula were prepared in PBS, and actual challenge concentration was confirmed by colony counts of dilutions spread plated on brilliant green agar (BGA) plates containing 20µg NA and 25µg NO to inhibit the growth of other bacteria (McElroy et al., 1994).

Morphometric Analysis

In Expt. 2 and 3, cecal samples (n=10/group) were collected 6 days post-challenge with SE or ST for analysis of mast cell number and changes in lamina propria thickness. Immediately following cervical dislocation, cecum from each bird was excised and fixed in 10% neutral buffered formalin (NBF). Five cross sections from each sample were placed in a tissue cassette and stored in 70% ethanol solution. Samples were fixed, paraffin embedded and processed by routine methods. Duplicate slides of each block were cut (5 microns) and stained with hematoxylin and eosin for measurement of lamina propria thickness or 1% toluidine blue for quantitation of mast cells. Using a bright-field microscope under a 40X objective, metachromatically stained mast cells found in the lamina propria of the ceca were quantified from a total of three complete tissue sections/slide (Figure 1). The mast cell numbers from the three sections were averaged to obtain a mean count for each bird, which was then used to determine means for treatment and sampling time.

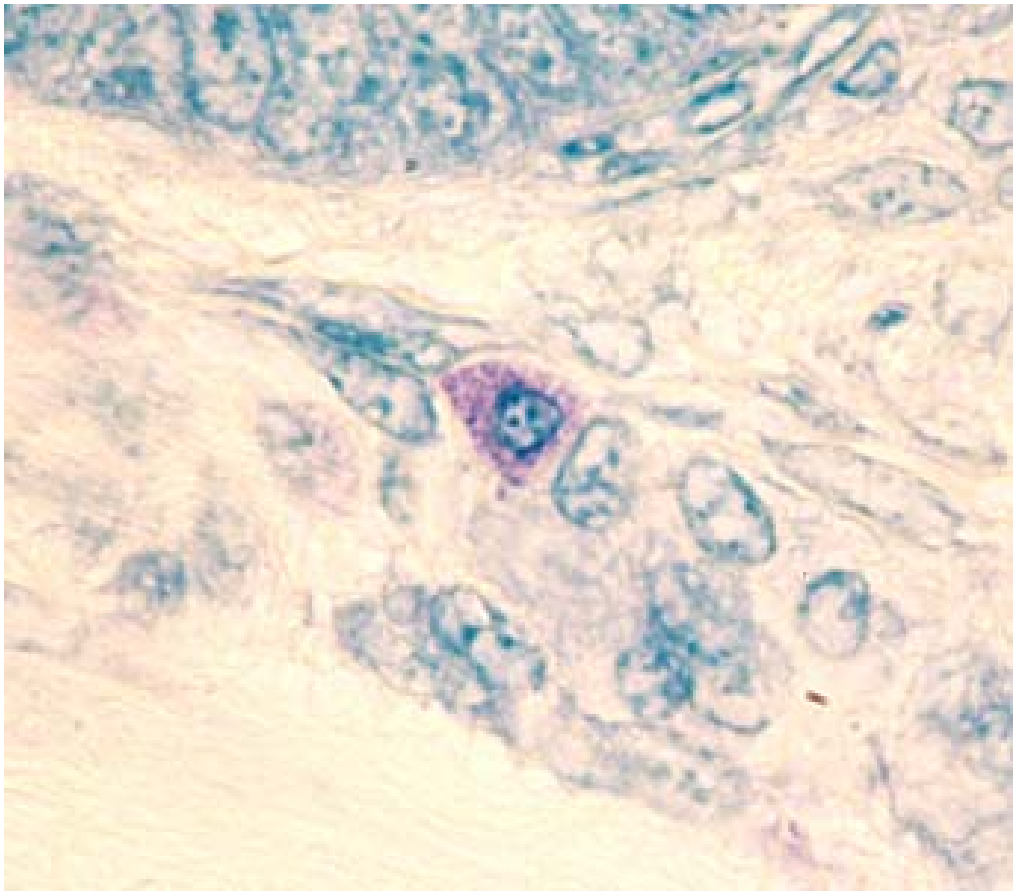

Fig. 1. Image of mucosal mast cell located in the lamina propria of the ceca. Mast cells were quantitated after being stained with toluidine blue.

For determination of lamina propria thickness, four measurements were obtained from the three highest quality tissue sections on each slide under 10X objective. Digital photomicrographs were taken and lamina propria thickness was determined by measuring the distance from the basement membrane to the muscularis mucosae using the Sigma Scan Pro5® software program (Figure 2).

Experimental Design

Three experiments were conducted to determine the effect of dietary CAP or CO administered during different phases of growout on host resistance to SE or ST. Expt. 1 was conducted to evaluate if inclusion of dietary CAP during the starter phase (days 1-16) changed the incidence of SE in challenged birds. Experiments 2 and 3 were designed to assess the effect of dietary spice grade CO in the finisher phase (days 29-37) or entire growout (days 1-35), on SE or ST incidence, number of colony forming units of SE or ST, lamina propria thickness, and mast cell number. On each day of sampling, birds were sacrificed by cervical dislocation. In all experiments conducted, liver, spleen, and cecal tonsils were aseptically removed to determine *Salmonella* isolation rate. One cecum from each bird was removed and immediately placed into NBF for histological analysis (Expt. 2 and 3) as described above. Cecal contents were collected for SE or ST enumeration (Expt. 2 and 3).

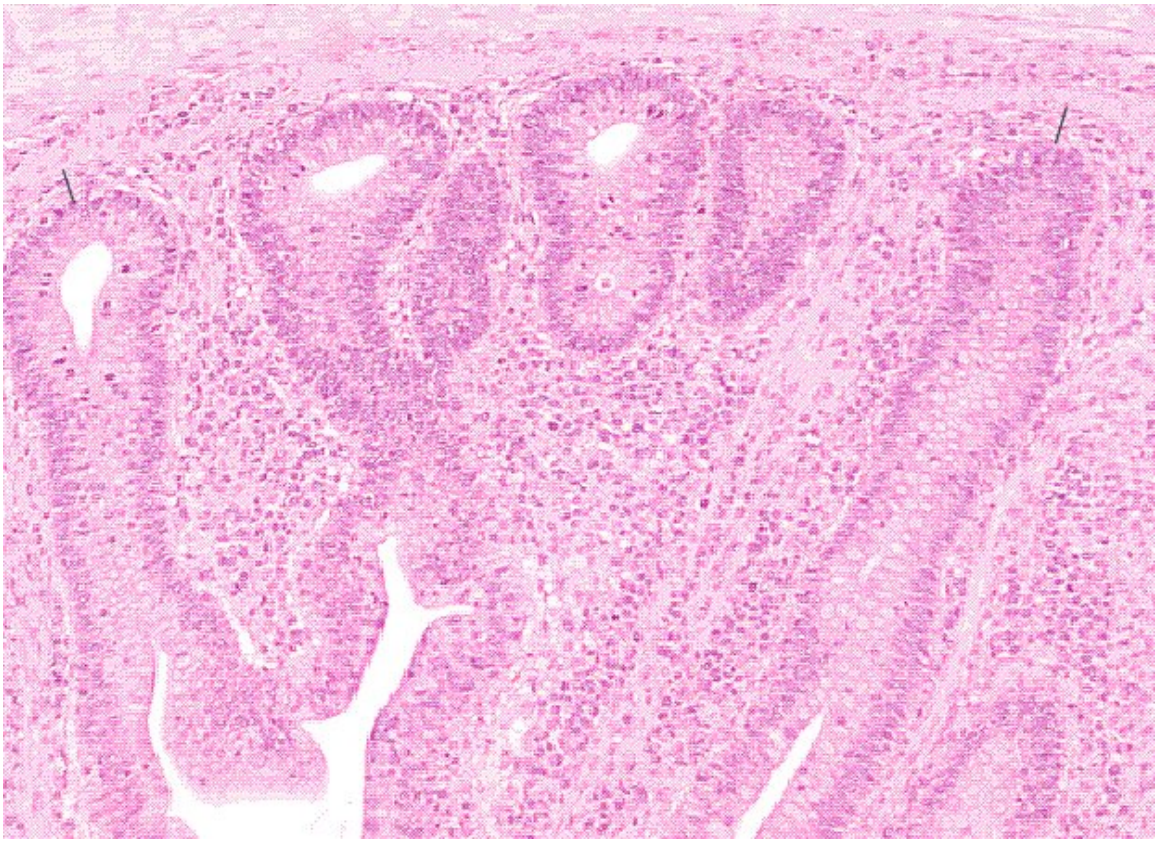

Fig. 2. Image of a cecal section 6 days post-challenge. Lamina propria thickness was measured from the basement membrane of the crypt to the muscularis mucosae as indicated by the black lines.

Salmonella Culture

For recovery of SE, internal organs were cultured as previously reported (McElroy et al., 1994). Briefly, samples of liver and spleen were aseptically collected, minced, and placed into tetrathionate broth as a single sample for each bird. For recovery of SE or ST, cecal tonsils were similarly collected and cultured separately from liver and spleen. Organs were incubated in tetrathionate broth for 24h at 37°C, gently agitated, and then streaked with a loop onto a BGA plate. Plates were subsequently incubated for 24h at 37°C. Plates were then examined for the presence of lactose-negative, NO/NA resistant colonies of SE or ST. For SE or ST enumeration, 0.25g of cecal contents were aseptically collected into tubes containing 2.25 ml of sterile Butterfield's diluent, and then serially diluted. A 100µl aliquot of each sample dilution was spread on a BGA plate and incubated at 37°C for 24h as previously reported (McElroy, et al., 1994). Suspect colonies were confirmed with lysine iron and triple sugar iron agar slants for observation of H_2S production, and sugar fermentation.

Experiment 1

Straight-run CobbxCobb broiler chicks were obtained from a commercial broiler hatchery on day-of-hatch. On day 1, birds were divided into one of four treatment groups (n=60/group); 0ppm CAP diet (non-challenged), 0ppm CAP diet (SE challenge), 10ppm CAP diet (non-challenged), and 10ppm CAP diet (SE challenge) to evaluate the effects of dietary administration of CAP (Sigma #3403, 60% capsaicin) during the starter phase following challenge with SE on day of age. Capsaicin powder was mixed in with feed after it was crumbled. Birds were challenged with SE on day of age by a horizontal transmission model in which a 1:2 ratio of inoculated to contact chickens was used. Birds inoculated received a *per os* gavage (0.5 ml) of SE of $1x10^4$ CFU/chick. On day 16 post-challenge, intact ceca, liver, and spleen were aseptically removed from each bird and processed as previously described.

Experiment 2

Straight-run broiler chicks (CobbxCobb) were obtained from a local commercial hatchery on day-of-hatch. On day 1, birds were divided into pens (n=30/pen) and reared in separate rooms according to treatment group; 0ppm CO diet (no challenge), 0ppm CO

diet (SE challenge) 5ppm CO diet (no challenge), 5ppm CO diet (SE challenge), 20ppm CO diet (no challenge), and 20ppm CO diet (SE challenge). For days 1-29, all birds were provided with 0ppm CO starter (days 1-16) and grower (days 17-31) diets. Dietary CO (5ppm or 20ppm) was initiated on day 30, and birds were challenged with SE ($1x10^8$ CFU/bird) by horizontal transmission on day 31. On day 6 post-challenge, birds were killed by cervical dislocation, and intact ceca and samples of liver and spleen were taken from each experimental animal. Cecal contents were obtained for CFU enumeration, and cecal tissue samples were taken for morphometric analysis as described above.

Experiment 3

Experiment 3 was conducted to evaluate the effect of prophylactic treatment with dietary CO on host resistance to SE or ST during a full growout period (day 1-37). Straight-run chicks (CobbxCobb) were obtained and divided into three experimental groups (0ppm, 5ppm, and 20ppm CO) with three replicates of each (20 birds/replicate). On each challenge day (day 14 and 29), birds (n=10) were selected from each replicate to comprise a total of 9 dietary treatment groups (n=30/group); 0ppm CO diet (no challenge), 0ppm CO diet (SE challenge), 0ppm CO diet (ST challenge), 5ppm CO diet (no challenge), 5ppm CO diet (SE challenge), 5ppm CO diet (ST challenge), 20ppm CO diet (no challenge), 20ppm CO diet (SE challenge), and 20ppm CO diet (ST challenge). Birds were transferred to pens with fresh pine shavings and challenged by horizontal transmission with SE or ST (day 14 challenge: $1x10^6$ CFU/ml, day 29 challenge: $1x10^8$ CFU/ml). On day 6 post-challenge, organ cultures, SE and ST enumerations and morphometric analyses were performed as described above.

Statistical Analysis

Enumerations were analyzed by ANOVA using the GLM procedures of SAS® (SAS Institute, 1998)[1]. Chi-square test of independence was used to determine significant differences in organ invasion or cecal colonization isolation rate between treatment groups. Differences in lamina propria measurements and mast cell counts were analyzed using orthogonal contrasts. Comparisons were made to determine the linear and quadratic effect of CAP alone or in combination with SE or ST challenge, the effect of

[1] SSPS Inc., Chicago, IL

Salmonella (SE or ST), and the effect of mode of exposure to *Salmonella* (direct or contact). All results, unless otherwise stated, are reported as significant at $P \leq 0.05$.

RESULTS

Experiment 1

Table 1 shows the results from Expt. 1, which evaluated the effect of administration of 0ppm or 10ppm dietary CAP for 16 days following challenge at day of age with SE. The therapeutic use of 10ppm CAP in the starter diet resulted in increased L/S invasion and cecal colonization with SE as compared to the incidence level in controls (0ppm CAP).

Experiment 2

Experiment 2 was conducted to evaluate the effects of 0ppm, 5ppm or 20ppm CO administration and SE challenge within a 24 hour period during the finisher growth phase. Dietary administration of 5ppm CO increased the number of SE positive ceca as compared with controls (0ppm CO) or birds receiving 20ppm CO (Table 2). However, while a trend toward increased number of positive samples was observed in L/S under CO influence (5ppm or 20ppm), no significant differences were found. Quantitation of cecal SE CFU indicated 1.05-log and 1.33-log fewer CFU under the dietary influence of 5ppm and 20ppm CO, respectively as compared to controls. No difference was observed in lamina propria thickness due to *Salmonella* or CO treatment alone. An interaction was observed ($P<0.005$) however, in birds that were administered both CO and SE challenge. A linear decrease in lamina propria thickness occurred with an increase in dietary CO concentration in birds challenged with SE. The opposite effect was observed in non-challenged birds receiving 0ppm, 5ppm or 20ppm CO. Quantitation of mast cell numbers in the lamina propria showed no differences between SE challenge or CO treatment, alone or in combination.

Experiment 3

Experiment 3 allowed observation of the effects of dietary CO administration over an extended time period i.e., 14 or 29 days of 0ppm, 5ppm or 20ppm CO. Two strains of *Salmonella*, SE and ST, were used, and the effects of prophylactic CO treatment on *Salmonella* challenge were evaluated. Number of cecal positives for SE in birds challenged on day 14 was decreased with CO incorporated in the diet at 5ppm or 20ppm as compared to control (Table 3). No differences were seen in L/S SE positive number with challenge on day 14. In contrast to aforementioned results, there was no

difference between treatments in SE cecal positive isolation rate in birds challenged on day 29. However, a significant decrease in SE positive L/S samples from birds receiving 20ppm CO was observed. Number of ST ceca positives from challenge on day 14 was found to decrease significantly in birds administered 5ppm CO as compared to control or 20ppm fed broilers. However, no differences between dietary treatment groups were observed with regard to ST cecal positive isolation rate in birds challenged on day 29. 1.1-log fewer SE CFU were found in birds challenged on day 14 receiving 5ppm CO administration as compared to control (0ppm CAP). Administration of 20ppm CO however, resulted in approximately the same number of SE CFU as found in birds not receiving CO. Birds challenged on day 29 demonstrated 1.08-log and 1.39-log fewer SE CFU in the ceca when administered either 5ppm or 20ppm CO, respectively. *Salmonella typhimurium* challenged birds demonstrated different results. Both challenge time points had approximately 1-log greater ST CFU numbers when fed 20ppm CO. Similarly, birds challenged on day 14 exhibited significantly higher ST CFU numbers when fed 20ppm CO as compared to 5ppm CO. However, neither level of CO administration exhibited CFU numbers different from 0ppm CO fed birds.

Significant effects observed in lamina propria thickness were due to interactions between CO treatment and *Salmonella* challenge (Table 4). A quadratic interaction ($P<0.05$) was observed between groups receiving SE challenge on day 14, and CO administration as compared to groups administered dietary CO only. Administration of CO in non-challenged birds was associated with decrease in lamina propria thickness in 5ppm fed birds, while lamina propria thickness increased approximately equal to that of 0ppm fed birds with 20ppm dietary CO. In contrast, birds receiving SE challenge exhibited increased lamina propria thickness when fed 5ppm CO, which was not further increased when the CO concentration was increased to 20ppm. No interaction was observed with birds challenged with ST on day 14. With challenge on day 29, both linear ($P<0.01$) and quadratic ($P<0.0005$) interactions were observed with CO level and SE or ST challenge as compared to non-challenged birds. Lamina propria thickness decreased with 5ppm CO administration and SE or ST challenge, while with 20ppm CO administration, the lamina propria thickness was approximately equal to that of 0ppm in both SE and ST challenged birds, but increased as compared to 0ppm, non-challenged

birds. In contrast, in the presence of dietary CO with no SE or ST challenge, lamina propria thickness increased with 5ppm CO then decreased with 20ppm CO. No differences were seen in mast cell number between any treatment group (Table 4).

Table 1. Effect of 10ppm dietary Capsaicin (days 1-16) on day of age *Salmonella enteritidis* (SE) challenge in broiler chickens.

	SE Incidence[A]	
Capsaicin	**Liver/Spleen**	**Cecal Tonsils**
0ppm	6/60(10)	22/60(36.7)
10ppm	20/56(35.7)*	50/56(89.3)*

[A]number positive/total number (% positive).

*Indicates significant difference from control at P<0.05.

Table 2. Effect of 0ppm, 5ppm or 20ppm dietary Capsaicin oleoresin (CO) in the finisher growth phase (days 30-37) on *Salmonella enteritidis* (SE) challenge (day 31) in broilers.

		SE Incidence[A]		Cecal Lamina Propria Thickness (mm)*		Cecal Mast Cell Number	
Capsaicin	**SE Cecal CFU[B]**	**Liver/Spleen**	**Ceca**	**No Challenge**	**SE**	**No Challenge**	**SE**
0ppm	1.71±0.48[C]	8/29(27.6)	22/29(75.9)	0.0111 ± 0.0017[C]	0.0155 ± 0.0016	15.73±3.99[C]	17.55±4.20
5ppm	0.66±0.35	14/30(46.7)	29/30(96.7)**	0.0135 ± 0.0017	0.0125 ± 0.0016	14.56±3.99	13.20±3.99
20ppm	0.32±0.25	13/29(44.8)	22/29(75.9)	0.0165 ± 0.0016	0.0110 ± 0.0016	16.53±3.99	14.16±3.99

[A]number positive/total number (% positive).

[B]Colony forming units.

[C]Values are means ± SEM.

*Indicates linear interaction of Capsaicin and SE at P<0.005.

**Indicates significant difference from control at P<0.05.

Table 3. Effect of 0ppm, 5ppm or 20ppm dietary Capsaicin oleoresin (CO) (days 1-14 or days 1-29) on *Salmonella enteritidis* (SE) or *Salmonella typhimurium* (ST) (day 14 or day 29 challenge) incidence and colonization in broilers.

		Cecal CFU[A]		SE Incidence[B]		ST Incidence[B]
Challenge	**Capsaicin**	**SE**	**ST**	**Liver/Spleen**	**Cecal tonsils**	**Ceca**
day 14	0ppm	2.25±0.52[C]	0.49±0.26	11/30(36.7)	23/30(76.7)	8/30(27.0)
	5ppm	1.15±0.36	0	5/30(16.7)	12/30(40.0)*	2/30(6.7)*
	20ppm	2.09±0.05	1.42±0.42	8/30(26.7)	15/30(50.0)*	10/30(33.3)
day 29	0ppm	3.38±0.30	1.15±0.36	18/27(66.7)	26/27(96.3)	12/30(40.0)
	5ppm	2.30±0.54	1.23±0.37	21/30(70.0)	27/30(90.0)	16/29(55.2)
	20ppm	1.99±0.48	2.11±0.42	12/30(40.0)*	24/30(80.0)	16/30(53.3)

[A]Colony forming units.

[B]number positive/total number (% positive).

[C]Values are means ± SEM.

*Indicates significant difference from control at P≤0.05.

Table 4. Effect of 0ppm, 5ppm or 20ppm dietary Capsaicin oleoresin (CO) (days 1-14 or days 1-29) and *Salmonella enteritidis* (SE) or *Salmonella typhimurium* (ST) challenge (day 14 or 29) on cecal lamina propria thickness and mast cell number.

		Cecal Lamina Propria Thickness (mm)[A]			Cecal Mast Cell Number[A]		
Challenge	**Capsaicin**	**No Challenge**	**SE**	**ST**	**No Challenge**	**SE**	**ST**
day 14*	0ppm	0.0084±0.0007	0.0077±0.0007	0.0095±0.0007	19.76±4.77	14.53±3.56	15.95±3.37
	5ppm	0.0077±0.0006	0.0092±0.0006	0.0088±0.0006	19.76±4.77	21.00±3.37	23.22±3.37
	20ppm	0.0092±0.0006	0.0091±0.0006	0.0086±0.0006	25.24±2.55	18.16±3.56	19.40±3.37
day 29**	0ppm	0.0113±0.0012	0.0115±0.0013	0.0123±0.0012	16.83±7.51	20.33±5.60	22.08±5.31
	5ppm	0.0169±0.0012	0.0113±0.0012	0.0091±0.0012	29.56±7.51	23.90±5.31	29.22±5.31
	20ppm	0.0008±0.0013	0.0123±0.0012	0.0115±0.0012	25.23±7.51	17.12±5.45	23.75±5.31

[A]Values are means ± SEM.

*Indicates significant differences in lamina propria thickness with quadratic interaction of CO and SE at $P<0.05$

**Indicates significant differences in lamina propria thickness with linear and quadratic interaction of CO and SE or ST at $P<0.05$.

DISCUSSION

Results from these studies suggested that the duration of CAP administration, CAP concentration, CAP chemical form, bird age, and *Salmonella* serotype all likely contributed to the differences observed. Previously, McElroy et al. (1994) found that dietary CAP administered continuously for 42 days of broiler growout, with challenge occurring on day 21, 28, or 42, decreased the recovery incidence for SE positive organs. However, while our laboratory found similar results in Expt. 3, our first two experiments demonstrated the opposite effect. It is possible that these differences were due to a change in the chemical form of CAP used or the sequence of CAP administration and challenge with *Salmonella.* In Expt. 1, purified CAP was used in purified form (60% capsaicin), while in Expt. 2 and 3, spice grade CO (1.08% capsaicinoids) was used and mixed into the fat source prior to pelleting of feed. While not known, the capsaicinoid derivatives may be different between the two compounds, CAP and CO, used for these studies. Furthermore, it is possible that the properties or concentration of capsaicinoids in CO were changed due to exposure to heat during the pelleting process further contributing to differences seen between the experiments. It is important to note that since CO was composed of only 1.08% capsaicinoids, other components in the oleoresin could also have contributed to observed results. Also, Expt. 1 and 2 examined the effects due to CAP or CO administration and subsequent challenge within 24h while Expt. 3 examined the effects of a minimum of 14 days of dietary CO exposure before challenge with *Salmonella*. Reduction of *Salmonella* positive organs as found by McElroy et al. (1994) and the current Expt. 3 could be a result of alterations in the gut from treatment with CAP prior to challenge with *Salmonella*. Since reductions in L/S and ceca positives were observed in studies with prophylactic CAP treatment, it is postulated that the presence of CAP in the gut could cause morphological changes. Consequently, changes such as thickening of the mucus lining or alteration of enterocyte maturation or protein synthesis, could possibly inhibit *Salmonella* invasion or alter the host's immune response. To attempt to elucidate some mechanism of action, we examined cecal CFU numbers as well as the histological and morphological changes.

In both Expt. 2 and Expt. 3, birds challenged with SE had approximately 1-log fewer CFU when receiving 5ppm CAP. In Expt. 2, a linear trend was observed showing

fewer CFU as CO concentration increased from 5ppm to 20ppm. Under simultaneous exposure to CO and SE challenge in Expt. 2, a 1.05-log or 1.39-log decrease in CFU was observed under the influence of 5ppm or 20ppm CO, respectively. While this would allow consideration that the action of CO is bactericidal, the results could also be indicative of a host response to CO, *Salmonella*, or an interaction of both. In Expt. 3, results were conflicting between the two serotypes of *Salmonella*, SE and ST. Birds challenged with SE on day 14 after receiving dietary 20ppm CO since day of age exhibited no change in CFU compared to birds not receiving CO at all, while 5ppm exposure resulted in 1.1-log decrease. *Salmonella typhimurium* seemed to be unaffected by inclusion of CO as indicated by approximately equal or increased CFU with 5ppm or 20ppm inclusion, respectively. It is also important to note that birds inoculated with ST and administered 5ppm CO demonstrated no differences in CFU compared with control groups. In contrast, under the influence of 20ppm CO, 1-log greater CFU numbers were observed at both challenge time points. This lack of decrease in CFU number with the higher concentration of CO does not correlate with the decreased SE isolation rate seen with 20ppm dietary CAP leading authors to speculate that a desensitization of neurons in the gut occur with higher concentration of or longer exposure to CO. Also, with reports from Tellez et al. (1993) of decreased cecal pH in the presence of CAP, bacteriostatic (not bactericidal) effects could be induced.

It is interesting that the observations from SE inoculated birds are opposite of those from ST inoculated birds. However, it is well understood that microbial pathogenicity is due to differences in virulence factors between serotypes (Suzuki, 1994), and this could be a contributor to differences observed between results from challenge with SE or ST in these studies. The previously reported effects of CAP on small intestinal morphology, such as increased lamina propria thickness and epithelial cell proliferation (Tellez et al, 1993), leads authors to speculate that CAP treatment could decrease the ability of SE to invade and colonize in the tissue. It has also been observed that mucus production from goblet cells increases in the presence of CAP (Hoffmann et al., 2002), which is known to provide a barrier against bacterial invasion (Albanese et al., 1994; Hoffmann et al., 2002). Also, an increase in *Salmonella* isolation rate was observed in the present studies under therapeutic CAP treatment, which could be a result

of increased invasion aided by a diminished mucus layer on the epithelial surface in the presence of bacteria, a decrease in intestinal pH (Tellez et al., 1993), or a change in the membrane permeability caused by the presence of CAP (Govindarajan and Sathyanarayana, 1991). These evidences further point to the possibility of a host effect in the presence of CAP leading to decreased *Salmonella* isolation rate and subsequent bacterial proliferation.

Lamina propria thickness was not affected by treatment with CAP or challenge with *Salmonella* alone, however, interactions were observed between birds fed CAP and challenged with *Salmonella* as compared to non-challenged, CAP fed birds. Previous evidence of increased lamina propria thickness in CAP fed birds (Tellez et al., 1993) led researchers to conclude that there was a direct correlation between presence of CAP and lamina propria thickness. The present studies, however, demonstrate that differing lamina propria thickness in birds fed the same concentration of CO was dependent upon whether or not they were challenged with *Salmonella*. It is interesting to note that birds challenged on day 14 demonstrated no differences in lamina propria thickness when given 0ppm or 20ppm CO treatment as compared to non-challenged birds of these dietary treatments. However, birds given a 20ppm CO diet and challenged with SE or ST on day 29 exhibited increased lamina propria thickness as compared to non-challenged, 20ppm CO fed birds, but no differences were observed when compared to 0ppm treated, ST or SE challenged birds. Also, 5ppm CO increased lamina propria thickness in SE challenged birds on day 14, while it decreased lamina propria thickness in both SE and ST challenged birds on day 29. Earlier reports from Manning et al. (1994) demonstrate that heterophil infiltration, villus height, and crypt depth were significantly increased in the presence of SE for 24h or CAP for 21 days as compared to non-challenged, 0ppm CAP fed birds. This differential effect could be related to dose and immuno-modulatory changes, such as infiltration of effector cells, occurring in the host with longer exposure to *Salmonella*.

Mast cell numbers varied between treatment groups, however, no significant differences were found. Mast cells are known to release chemoattractants resulting in recruitment of neutrophils and bacterial phagocytosis or killing of opsonized bacteria (Feger et al. 2002). All mast cell measurements were obtained from samples taken 6

days post-inoculation in the present experiments. Morphological changes due to *Salmonella* in calves have been observed as early as 15 minutes post-inoculation (Santos et al., 2002) suggesting a rapid host response post-inoculation with *Salmonella*. It has been observed that murine macrophage production of TNF-α increases in response to LPS as early as 2 to 6 hours post-inoculation (Arnold et al., 1993), however, Yu et al. (1998) found that dietary CAP had no effect on phagotcytic activity of peritoneal macrophages, but secretion of TNF-α increased in mice fed 20ppm CAP. This evidence could mean that increased TNF-α production could be a result of an immediate response to *Salmonella* resulting in mast cell degranulation. In future experiments, it may be advantageous to collect samples at earlier time intervals post-inoculation in order to obtain a more complete picture of any possible mast cell response to *Salmonella* colonization.

Another variable to consider is the secretion of SP resulting from exposure to CAP. It is reported that subcutaneous injection of CAP has been found to decrease SP production resulting in decreased IgM and IgG production (Eglezos et al., 1990), however, dietary CAP (20ppm) treatment in mice induced lymphocyte proliferation, immunoglobulin production and TNF-α secretion (Yu et al., 1998), which could result from induced SP secretion due to dietary CAP treatment (Surh and Lee, 1996). Substance P induces the degranulation of mast cells (Shanahan et al., 1985), which could aid in clearance of bacteria from the gut through phagocytosis by mast cells and chemotaxis of other immune cells such as macrophages and neutrophils. The currently reported procedures only allowed quantitation of mast cells through staining of the granules, therefore, if SP levels were increased causing mast cell degranulation, the exact number of mast cells present would have been impossible to quantitate accurately. Future investigations to measure SP levels in the gastrointestinal tract under the influence of CAP are warranted.

Also, T lymphocytes, B lymphocytes and macrophages have been shown to increase in the oviduct mucosa following inoculation with SE (Withange et al., 1998). Similarly, significant increases in antibody titers were detected in bile (IgA and IgG) and the intestinal mucosa and serum (IgG and IgM) of chickens infected with ST (Brito et al., 1993). These present experiments did not evaluate changes in lymphocytes or

macrophages. Although dietary CAP is shown to increase immune effects, such as immunoglobulin production, morphological alterations could negate these immunological effects by allowing increased colonization by *Salmonella*.

Future studies evaluating the immediate response of CAP or CO administration on the presence of mucosal mast cells, as well as serum and macrophage receptor studies to evaluate lymphocyte populations could provide more evidence for the action of CAP. Currently reported results have provided little evidence for the mechanism of action of CAP, however, speculations that a host effect to CAP is involved appear to be correct. This mechanism remains ill-defined due to the many variables involved, such as duration of CAP exposure, CAP dose, CAP chemical form, bird age, and *Salmonella* serotype. However, due to these preliminary results, it is apparent that prophylactic treatment with CAP does decrease *Salmonella* positive ceca and L/S organs, and an interaction between CAP and *Salmonella* results in morphological alterations. Further defining results of CAP dose, histological and morphological alterations could provide evidences needed to further understand the host response to CAP.

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