

# Nutrients, Nutritional State and Small Intestinal Microbiota

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## ***Gastro-Intestinal Tract (GIT) Barrier Function***

### **Non-Immune Defenses**

The intestine is an organ that must meet two seemingly incompatible goals: to maximize nutrient uptake and to minimize antigenic insult while tolerating the presence of indigenous microbiota and other antigens introduced by the presence of feed within the intestinal tract. Both of these functions require a number of multifaceted interactions between numerous physiological systems such as the physical GIT barrier and the GIT immune system and the indigenous microbiota. The synchronization of these systems is crucial to maintaining nutrient uptake, utilization, and animal health as well as defending against constantly adapting bacteria and other antigenic insults. Because the interactions between various components of the barrier function of the GIT are so complicated, there is much to be learned about gut barrier function, and researchers are just beginning to grasp the complexities of the gut.

Among the first line of non-immune defenses associated with gut barrier function is the mucus layer overlying the epithelial cells. This layer is composed of neutral, acidic, and sulphated mucin glycoproteins containing a diverse array of carbohydrate structures which are created and secreted by goblet cells located along the villi within the intestinal epithelium (Forstner and Forstner, 1994). In rats, the mucus layer can be divided into two distinct layers; a loosely adherent layer of mucus which lubricates the intestine and a firmly adherent layer of mucus which may serve as a stable protective barrier. Thickness of the two layers varies by section of the intestine in rats but it is unknown if chickens have two layers of mucus, and removal or sloughing of the outer loosely adherent mucus layer causes increased mucus secretion to replenish the outer layer (Atuma et al., 2001).

Many functional properties have been ascribed to intestinal mucins, such as lubrication of intestinal surfaces, trapping and neutralizing bacteria, detoxification of heavy metal binding, interactions with the intestinal immune system, acting as a diffusion barrier for nutrients and macromolecules, and protecting the underlying epithelial cells (Forstner and Forstner, 1994). Because the mucins contain a diverse array of carbohydrate structures, they provide numerous potential attachment sites for commensal and pathogenic bacteria and also serve as a colonization niche for intestinal bacteria found within the intestine (Sonnenburg et al., 2004). Moreover, the tightly adherent mucus layers bind with mucin binding protein receptors on the underlying epithelial cells, thus preventing bacterial access to some epithelial receptors (Slomiany et al., 2001). However, by aiding potential pathogens in gaining an attachment site, the mucus layer may also allow pathogens to migrate through the mucin towards the epithelium where colonization or release of toxins may occur. Protective factors are in place, however, as both mucin layers are known to accumulate bacteriocidal and bacteriostatic compounds, and secretory immunoglobulin A, compounds which are capable of neutralizing or killing the trapped bacteria. Secondly, as the loosely adherent mucus layer is sloughed, it also traps and carries the resident or invading bacteria, thus removing the trapped bacteria from the GIT. Therefore, although mucus is generally seen as an important factor in maintaining a strong intestinal barrier, it is difficult to predict exactly how the mucus layer will inhibit or aid specific pathogens in the invasion process, as many factors influence the outcome of bacterial-mucin interactions, such as

mucin composition, quality, quantity, digesta flow, and gut motility (Forstner and Forstner, 1994).

## **Immune Defenses**

Non-immunologic contributions to gut barrier function are very important, yet the immune system cannot be overlooked. Researchers have estimated that the intestinal tract contains greater than 70% of all immune cells found within the body and that nearly one-fourth of the intestinal mucosa is composed of lymphoid tissue (Kagnoff, 1993). Thus, the intestinal immune system has been given its own designation as GALT, or gastrointestinal-associated lymphoid tissue, and contributes greatly to gut barrier function on a day-to-day basis.

The protection that is provided by GALT can be broken down into two categories: innate and adaptive immunity. Innate immunity is defined as a non-specific immune defense and consists of physical barriers such as epithelial cells or secretions such as mucus, antibacterial peptides such as defensins, lysozymes, or lactoferrin, and phagocytes and macrophages which engulf and destroy bacteria. None of these factors specifically target invading pathogens, but instead provide an initial defense against them and enhance the mechanisms of adaptive immunity. Adaptive or acquired immunity is significantly more specific to individual antigens, which are defined as any substance that the body recognizes as foreign, and is characterized by the development of T cells, B cells, and antibodies that are antigen-specific (Muir, 1998).

One of the primary components of the GALT is the lamina propria of the intestine, which is the connective tissue that underlies the epithelium of the gut. Highly vascularized and richly innervated by the enteric nervous system (Gaskins, 1997), the lamina propria contains sizeable populations of immune cells such as the B and T lymphocytes, immunoglobulins, macrophages, mast cells, and plasma cells, among others (Kagnoff, 1993). Additionally, intraepithelial lymphocytes (IEL) reside within the epithelial layer of the intestine, and produce numerous cytokines that mediate the immune response.

Immune cells within the Peyer's patch and lamina propria are presented with the sampled bacteria and initiate a chain of events that help the immune system recognize and respond to the bacteria. Among these responses is the plasma cell production of secretory immunoglobulin A (IgA), an antibody that is specific to the bacteria it was presented with, and an immunoglobulin which is recognized as the main immunologic intestinal defense against the attachment of bacteria to the epithelial layer (Spitz et al., 1996). Secretory IgA is secreted into the intestinal lumen and provides protection to the intestine by attaching to bacteria, neutralizing the bacteria and preventing the bacteria from adhering to and injuring or destroying epithelial cells. Furthermore, sIgA is also capable of killing the bacteria by presenting it to immune cells that will destroy the bacteria (Kagnoff, 1993). Large concentrations of sIgA exist within the lumen of the intestine and coat many of the bacteria within the lumen, thus contributing to maintaining gut barrier function. Additionally, the production of IgA within the intestine stimulates the production of memory T and B-cells, or cells that automatically recognize the specific bacteria in subsequent encounters. This recognition is advantageous to the animal because the immune systems is able to recognize and respond to the bacteria without the processing that was necessary during the first exposure, thus decreasing the response time (Kagnoff, 1993).

## ***Intestinal Microbiota***

The avian GIT accommodates an extensive and diverse indigenous microflora that is crucial to proper intestinal function and defense. The indigenous microflora of the mature avian intestinal tract is composed of 640 bacterial species and 140 different bacterial genera, 99.9% of which are obligate anaerobes (Moore and Holdeman, 1974; Apajalahti et al., 2004). Of the more than 640 species inhabiting the mammalian GIT, 99% of the indigenous microflora is composed of 30 to 40 bacterial species (Moore and Holdeman, 1974), and these bacteria, along with the remaining

1% of other species, occupy all available niches or habitats and establish a stable ecosystem within the GIT (Berg, 1996). Furthermore, bacterial cells in the GIT not only outnumber eukaryotic cells in the host body by a factor of 10 (Simon and Gorbach, 1984), but also contain 50 to 100 times as many genes as the host body (Hooper and Gordon, 2001).

The distribution of indigenous microflora within the avian GIT is not random, but organized qualitatively and quantitatively along vertical and horizontal regions in the GIT (Berg, 1996). The vertical distribution refers to the distribution of bacteria from the oral cavity to the colon, and concentrations of bacteria are vastly differently among different sections of the GIT. Furthermore, bacteria are distributed horizontally along the GIT as well, and occupy the intestinal lumen, mucus lining, crypt spaces, and adhere to the epithelial cells (Roze et al., 1982). Thus, each segment and horizontal layer of the GIT harbors its own specific bacterial community, a phenomenon which may be attributed to environmental factors in the intestine, such as nutrition, bile salts, oxygen concentration, and intestinal pH of the different intestinal segments (van der Wielen et al., 2002).

The resident intestinal microbiota provides a wide range of benefits to the host. Generally, the most beneficial and perhaps important role of the microbiota is colonization resistance, or the ability of the microbiota to inhibit the colonization of the gut by exogenous pathogenic bacteria (Berg, 1996). Inhibition of potential pathogens by the indigenous microbiota is achieved by numerous mechanisms, including competition for epithelial attachment sites and nutrients, and the production of bacteriocins, short chain fatty acids, and modified bile acids that are inhibitory to pathogens (Rolfe, 1997).

The indigenous microbiota also provides other benefits to the intestine. Indigenous bacteria contribute to the development of the young animal's GIT and also increase the host's resistance to infection (Gaskins, 2003). Specific short chain fatty acids produced by the fermentation processes of the microbiota serve as a major energy source to enterocytes in the colon (Guarner and Malagelada, 2003) and as stimulators of epithelial cell proliferation in the small and large intestine (Frankel et al., 1994), suggesting a role in maintaining the intestinal integrity, and therefore, preventing disease. Additionally, the indigenous microbiota is capable of converting dietary pre-carcinogens and carcinogens into non-carcinogenic compounds (Berg, 1996). Lastly, indigenous microbiota are known to synthesize vitamins and to increase the absorption of calcium, magnesium, and iron (Hill, 1997; Miyazawa et al., 1996).

In birds, the largest concentrations of bacterial populations are found in the distal ileum, ceca, and colon, although bacterial populations are also found in the crop, proventriculus, and gizzard as well. Bacterial populations within the crop are dominated by the lactobacilli species, and may contain low concentrations of other organisms such as *Clostridium perfringens*, micrococci, staphylococci, and yeast (Sarra et al., 1985; Mead, 1997). The proventriculus and gizzard are relatively inhospitable to bacteria due to a low pH and rapid transit of feed and thus only a few species of bacteria are located within these organs (Mead, 1997). Among those bacteria reported to be found in the gizzard and proventriculus are *Lactobacillus* as well as low populations of *E. coli*, enterococci, and yeasts (Smith, 1965).

### **Vertical Distribution**

The duodenal and jejunal sections of the intestine contain relatively low microbial densities which increase considerably at the distal ileum, ceca, and colon to densities of  $10^{11}$  bacteria per gram (wet weight) of cecal content (Savage, 1977; Mead, 1997). Analysis of the ileum of 6-week-old broiler chickens yielded an average count of  $10^8$  to  $10^9$  bacterial cells per gram of ileal digesta and  $10^{11}$  bacterial cells per gram of ileal mucosa, with 95% of the bacterial cells identified as gram positive (Gong et al., 2002). In this study, Lactobacilli, *E. cecorum*, and butyrate-producing bacteria were the three major bacterial groups that were detected and identified in the ileum and ceca, though the ileal mucosa was found to have less bacterial diversity than the cecal mucosa. However, these findings are not in agreement with those of Zhu

et al. (2002), whose recent analysis of broiler chicken cecal content and mucosa determined that only four phylogenetic groups, *Clostridium leptum*, *Sporomusa* sp., *Clostridium coccooides*, and the enteric bacteria group, comprised 89% of all bacterial sequences isolated from the ceca. The remaining 11% of bacterial species were represented by the *Atopobium* group (3.6%), the *Bacillus-Lactobacillus-Streptococcus* subdivision (1.5%), *Bacteroides* group (1.9%), *Actinomyces* and relatives (1.3%), and *Pseudomonas* and relatives (0.7%).

At hatch, the intestinal tract of the chick is generally sterile. However, within hours of hatch, bacteria from the chicks' environment begins to colonize the intestinal tract, and within h after hatching, bacterial populations in the ileum and ceca can reach  $10^8$  and  $10^{10}$  CFU/g of digesta (Lev and Briggs, 1956; Apajalahti et al., 2002). However, the initial population is most likely composed of only a few species, namely the enteric, Lactobacillus, and Bifidobacterium groups (Zhu and Joerger, 2003). During the days following hatch, bacterial species compete with one another in an attempt to become established within the intestine. For example, lactobacilli appear by the fourth day and large populations of obligate anaerobes appear soon after (Mead and Adams, 1975). This process, known as ecological succession (Wilson et al., 1986), continues through stages until a stable climax bacterial population that closely resembles that of adult birds develops within the small intestine (Smith, 1965; Rolfe, 1997). At this point, researchers are uncertain as to when an adult population is established. Research by Lee et al. (2002) has indicated that populations of indigenous microbiota vary significantly with age up to 49 days, although Lactobacillus and Clostridium groups are found in the ileum at all ages up to 49 days. Additionally, van der Wielen et al. (2002) observed very young broilers had similar bacterial communities, but that the complexity or diversity of the community increases with age and becomes less similar between birds.

Interestingly, individual differences between birds may account for the differences observed up to 49 days of age. Denaturing gradient gel electrophoresis (DGGE) data from van der Wielen et al. (2002) suggests that birds from the same flock share bacterial population similarities of only 50%. These similarities are surprisingly small, as one would think that birds grown in the same environment would have greater bacterial population similarities. Nonetheless, the low microbiota similarities between birds may suggest that host factors, such as host immunology (Toivanen et al., 2001), host genotype, specific host receptors, or bacterial communication systems (Zoetendal et al., 2001) contribute significantly to the bacterial profile of the intestinal tract of individuals. Other research, however, would suggest that the bacterial similarities are greater than 50%. Knarreborg et al. (2002) demonstrated that ileal bacterial similarities were nearly 74, 72, 51, and 48% similar at 7, 14, 21 and 35 days of age. Using G+C profiling, Apajalahti et al. (2001) determined that the cecal G+C content of 6 individual chickens from one farm were similar. The discrepancies between the data may arise from the different lab analyses for each experiment or age differences.

## Horizontal Distribution

As mentioned previously, bacterial populations also inhabit 5 distinct microhabitats (Herias, 1998). Our knowledge of factors affecting horizontal populations and functionality in poultry specie is very limited and borne primarily from mammalian studies.

- 1) Epithelial cell surface – specific binding often mediated by organelles such as fimbriae and afimbrial adhesions.
- 2) Deep adherent mucus layer – more motile bacteria due to active chemotaxis. Includes spiral-shaped specie such as *Borrelia*, *Treponema*, and *Spirillum*.
- 3) Mucus layer – bacteria contain specialized organelles such as flagella, and are capable of active mucolysis due to proteolysis of the nonglycosylated regions of mucin glycopeptides, sialidases, glucosulfatases, and/or glycosidases. Specie include both commensal bacteria such as Bacteroides, Bididobacterium, and

Eubacterium as well as pathogenic bacteria such as *Salmonella typhimurium*, *Vibrio cholerae*, and *Helicobacter pylori*.

- 4) Bacterial biofilms – indigenous bacteria forming multiple layers in nutrient rich areas such as the oral cavity. Species in biofilms include those such as *Actinomyces* and *Streptococci*.
- 5) Intestinal lumen – bacterial species are much more transient due to digesta flow.

## **Nutritional Effects on Intestine and Microbiota**

### **Fiber Content and Cereal Source**

From gross morphology, we have known for some time that changes in dietary fiber composition can have profound effects on morphological changes in villus height and enterocyte migration rates in order to account for reduced nutrient density (Moran, 1985). This shift, however, comes at a cost through tissue maintenance. Other changes to the intestine, include an increase in mucin production and goblet cell volume and number, with these effects largely dependant upon non-starch polysaccharide composition and solubility (Satchithanandam et al., 1990; Lundin et al., 1993), as pectin and cellulose do not induce mucin discharge (Barcelo et al., 2000). This increase in mucin volume corresponds with the dissolution of large molecular weight non-starch polysaccharide complexes (arabinoxylans and  $\beta$ -glucans) and subsequent increase in intestinal viscosity when cereals such as wheat, rye, triticale, barley and oats are fed.

Mucin volume and composition can largely influence both the horizontal and vertical distribution of the microbiota in the GIT. For example, Apajalahti et al. (2001) determined that the addition of dietary wheat significantly increases the populations of cecal bacteria with a 50 to 55% G+C content but dramatically decreased the populations of bacteria with a G+C content of 60 to 69%. Furthermore, the researchers determined that feed manufacturing also affected intestinal bacterial populations, suggesting that feed mixing and processing influences intestinal bacterial populations. Further research by that same group (Apajalahti et al., 2004) indicated that corn-fed broilers had cecal bacterial populations favoring low G+C microbes (20-34%) while reducing the populations of higher G+C microbes (65-69%). Wheat-fed broilers exhibited a cecal bacterial population that was more abundant with the higher G+C microbes. Although G+C profiling does not reveal the identity of the bacteria, the researchers pointed out that bacteria with low G+C content are typically clostridia, enterococci, and/or lactobacilli, while bifidobacteria contains a higher G+C content.

Certain bacteria also can become virulent when exposed to different mucin compositions and viscosity. For example, vibrio that is virulent expresses flagella allowing for movement through the mucus layer more readily than avirulent forms of vibrio which are non-motile. Similarly, more virulent strains of *S. typhimurium* possess the ability to bind to sialomucins and produce sialidase versus those that are not. (Deplancke and Gaskins, 2001).

### **Dietary Fat and Short Chain Acids**

Dietary fat source also influences the composition of intestinal bacteria. Research by Knarreborg et al. (2002) has demonstrated that while lipid source did not affect total numbers of enterobacteria, the inclusion of soy oil decreased the number of *Clostridium perfringens* when compared with the inclusion of a mixture of dietary lard and tallow (1.5:1).

Short chain fatty acids (SCFA) produced by different bacterial species have been known for some time to have direct effects on enterocyte proliferation rates, particularly in the hind-gut where concentrations are considerably higher (Recchkemmer and Engelhardt, 1981). SCFA also have a distinct direct contribution to mucin secretion (but not synthesis or goblet cell number), largely due to osmolality gradients in the lower small intestine (Sakata and Engelhardt, 1981). This

effect may be specific, as increased mucus secretion has been noted in rats when given acetate, propionate, and butyrate individually, or as a mixture in a dose dependant manner; whereas lactate and succinate did not (Shimotoyodome et al., 2000).

Typically, the higher luminal pH in the lower GIT is above the pKa of most short-chain organic acids (i.e. favoring the protonated acid, or R-COOH; Dibner and Buttin, 2002). However, a pH gradient can exist through the mucus layer along the crypt/villus axis (Engelhardt et al., 1989). If the short-chain organic acid were placed in an pH environment below it's pKa, it would disassociate and easily penetrate the lipid membrane of most bacterial cells and become cytotoxic to the bacteria (Ricke, 2003). The question remains, however, as to what substrates cause the proper environment for pH shifts and microbial environments for this bactericidal action to occur. For example when considering metabolizable sugars, pH shift at the mucosal surface is known to be greater for glucose > fructose > mannose.

## Antibiotics

Numerous attempts have been made in an effort to reduce the presence of pathogens within the birds' GIT, both in an effort to reduce potential sources of carcass contamination and in order to reduce any negative effects the pathogens may elicit on the performance of the bird. In the past, direct-fed antibiotics have been the gold standard by which growth promotion and disease resistance were measured. Used since the 1940's, sub-therapeutic doses of antibiotics have enhanced animal performance by increasing growth, improving feed efficiency, favorably altering intestinal bacteria and reducing incidence of disease. The exact mechanisms by which these improvements occur, however, are still not fully understood. Currently, four mechanisms of growth promotion have been proposed by various scientists. Because early research has indicated that orally dosed antibiotics do not promote growth in germ-free chicks (Coates et al., 1963), each of these proposed mechanisms are based on the hypothesis that the presence of bacteria in the intestine reduces animal growth, and include hypotheses that: 1) antibiotics inhibit the occurrence of sub-clinical infections, 2) antibiotics reduce production of growth-depressing microbial metabolites, 3) antibiotics reduce the use of nutrients by intestinal microbes, and 4) antibiotics allow for enhanced uptake of nutrients because they have been shown to reduce the thickness of the intestinal wall (Visek, 1978; Anderson et al., 1999; Gaskins et al., 2002). Regardless of the fact that the exact mechanisms of antibiotic-mediated growth promotion are currently incompletely understood, most researchers support the theory that antibiotics reduce the overall numbers or diversity of gut bacteria, which may promote growth (Dibner and Richards, 2005).

Antibiotics do significantly influence the intestinal bacterial community, though research regarding the effects of antibiotics on the entire intestinal bacterial community is in its infancy. Unfortunately, much of the research that has examined the effects of antibiotics on intestinal bacterial communities has utilized classical microbiological analysis, methodology which employs bacterial plating to determine bacterial population shifts in the GIT. This method, while providing a wealth of information regarding the effects of antibiotics on bacteria, is severely limited by the lack of understanding of the intestinal bacterial requirements for growth and hence the lacking development of sufficient selective media to grow the various intestinal bacteria. Furthermore, the intestine harbors roughly 640 various species of microbiota that exist in a complex and ever-changing intestinal community, and plating is not applicable to many of these bacteria because they are currently uncultivable. In fact, recent research by Zhu et al. (2002) estimates that only 20 to 50% of all intestinal bacteria have been cultured in lab experiments, leading the researchers to suggest that molecular techniques are superior to conventional plating techniques in identifying intestinal bacteria and its response to antibiotics.

Currently, few studies have utilized molecular techniques to examine the effects of antibiotics on intestinal bacteria communities. Collier and coworkers (2003) observed that tylosin decreased duodenal and jejunal bacterial population diversity and that the bacterial communities of birds fed tylosin had greater similarities than those of birds not fed tylosin. The data also indicated

that tylosin had a tendency to decrease total bacterial concentration, and significantly decreased *C. perfringens*. Further research on mice by the same group (McCracken et al., 2001) has determined that the inclusion of 25ppm of the broad-spectrum antibiotic cefoxitin to the drinking water reduces the similarity of intestinal bacterial communities, but does not affect the number of bacterial species as measured by denaturing gradient gel electrophoresis (DGGE). Other research by Knarreborg et al. (2002) determined that when combined, avilamycin and salinomycin caused shifts in the species of *Lactobacillus* found within the broiler ileum, as well as reducing plate counts of *C. perfringens*. Further, these two antibiotics also caused a shift in the nucleotide sequence of the alpha-toxin gene produced by *C. perfringens* (Knarreborg et al., 2002). The question remains, however, if this sequence shift would reduce incidence of necrotic enteritis susceptibility or not.

In a comparison of bacitracin and salinomycin (Engberg et al., 2000), researchers concluded that neither antibiotic affected plate counts of broiler ileal anaerobic bacteria, lactose-negative enterobacteria, lactic acid bacteria, enterococci, or lactobacilli. However, both antibiotics decreased *C. perfringens* while bacitracin increased cecal lactic acid bacteria and salinomycin decreased the same bacteria in the ceca.

Data from numerous plating studies, combined with knowledge of individual antibiotic's modes of action, indicates that different antibiotics elicit distinctive responses in intestinal bacterial communities. For instance, different classes of antibiotics are known to inhibit or kill different types of bacteria by inhibiting bacterial protein and cell wall synthesis, disrupting the bacterial cell membranes, and interfering with bacterial enzymes (Lorian, 1996).

Bacitracin, which belongs to the polypeptide class of antibiotics, has bactericidal effects against gram-positive bacteria, and kills bacteria by forming a complex with and inactivating C<sub>55</sub>-isoprenyl pyrophosphate, which is a carrier of peptidoglycan precursors that are necessary for the synthesis of the bacterial cell wall (Stone and Strominger, 1971). Other research has indicated that bacitracin may also interfere with other bacterial cellular processes as well (Pollock et al., 1994). Bacitracin is often used in the poultry industry as a means of improving growth and feed conversion as well as controlling necrotic enteritis in birds. Interestingly, bacitracin has also been reported to reduce the energy maintenance requirement (Bronsch and Manner, 1988) and improve heat tolerance of laying hens (Manner and Wang, 1991). Numerous studies have reported positive growth responses to bacitracin in birds, and in 2000, bacitracin was used more frequently in starter and grower broiler diets than any other antibiotic (Chapman and Johnson, 2002).

In chickens, bacitracin has been shown to reduce populations of *Clostridium perfringens* (Hock et al., 1997; Engberg et al., 2000), an indigenous species of bacteria that is known to cause necrotic enteritis. Additionally, dietary bacitracin reduces intestinal enterococci populations (Barnes et al., 1978) and has bacteriocidal effects on populations of *Lactobacillus* spp. (Dutta and Devriese, 1992) and *Staphylococcus* spp. (Devriese, 1980). The effects of bacitracin on the intestinal bacterial community are not fully known at this point, but with the development of new molecular techniques to examine bacterial communities, it will be possible to better understand how bacitracin functions within the intestine.

Another antibiotic, salinomycin, has also been used frequently in poultry diets, and is typically used for its anticoccidial effects, improvement of gain and feed conversion (Waldroup et al., 1986), and prevention of necrotic enteritis (Knarreborg et al., 2002). Salinomycin, one of the most commonly used coccidiostats in the broiler industry (Chapman, 2001), is a bactericidal compound that causes bacterial and parasite death by interfering with the natural ion transport systems of the cells (Elsasser, 1984). When fed in the diet, salinomycin reduces populations of *Clostridium perfringens* (Knarreborg et al., 2002), *Lactobacillus* spp. (Rada et al., 1991), enterococci, and *Staphylococcus* spp. (Aarestrup et al., 1998). However, dietary salinomycin does not affect the frequency or magnitude of *Salmonella typhimurium* DT104 shedding (Scalzo et al., 2004). Much like bacitracin, however, there is little research regarding the effects of

salinomycin on intestinal bacterial communities, and it is apparent that further research is necessary to better understand the dynamics of different types of antibiotics and their effects on intestinal bacterial populations.

## Copper

Supplementation with copper well above nutrient requirements has been shown to improve the rate and efficiency of body weight gain in pigs and chickens (Cooke, 1981; Edmonds et al., 1985; Aoyagi and Baker, 1995; Pesti and Bakalli, 1996), though the underlying mechanisms of growth promotion are presently unclear. High concentrations of Cu were originally ‘touted’ as having benefits in prevention of crop mycosis. Indeed, field studies indicate it does have some merit, but reproducibility of induced crop mycosis in experimental conditions has had less than favorable results (Underwood et al., 1956). In fact, addition of up to 250 ppm Cu results in increased erosion to the lining of the gizzard (Fisher et al., 1973; Poupoulis and Jensen, 1976) and results in an “inhibition of normal fermentation” in the cecae of the chick (Jensen and Maurice, 1978). This observation has been confirmed in *in vitro* anaerobic digestion. In particular, volatile fatty acid production can be inhibited considerably due to reductions in microbial activity (Yenigun et al., 1996).

Many hypotheses regarding the mechanisms of copper-mediated growth promotion have been proposed. Zhu et al. (1994) has proposed several mechanisms, such as alterations in the indigenous bacterial communities, increased serum mitogenic activity, increased expression of growth hormone, increased secretion of neuropeptide, or increased levels as a component of a growth factor, that allow growth promotion. Aoyagi and Baker (1995) have also proposed that birds fed pharmacological doses of copper (250ppm from copper sulfate) may improve hemicellulose digestibility, which may improve growth.

Of these, the effect of copper on intestinal bacterial populations has been under recent investigation. Researchers hypothesize that copper favorably alters or inhibits certain bacterial populations within the intestinal milieu of bacteria, thus allowing for the improved growth rates, though this theory has yet to be fully proven. This theory is based on evidence that copper inhibits bacterial growth and has a toxic effect on many microorganisms. Copper toxicity can cause inactivation of bacterial enzymes and alterations in bacterial membrane integrity (Ohsumi et al., 1988) as a free cation (Zevenhuizen et al., 1979; Menkissoglu and Lindow, 1991). Additionally, another source of copper toxicity is membrane-bound copper production of hydroperoxide free radicals (Rodriguez-Montelongo et al., 1993), blocked functional groups of proteins, and the “open gate” system (Nies and Silver, 1995), in which cytoplasmic copper concentrations cannot be regulated and thus become toxic.

There is little data regarding the effects of copper on intestinal bacteria populations. Varel et al. (1987) determined that 125ppm dietary copper sulfate caused marked reductions in swine intestinal populations of *Streptococcus* spp. Furthermore, the authors observed nonsignificant reductions in *Staphylococcus* spp., *Bacteroides multicus*, and *Eubacterium limosum*. *In vitro* research has demonstrated that *Staphylococcus aureus* and *hyicus*, *E. coli*, and *Enterococcus faecalis* and *faecium* are sensitive to concentrations of copper ranging from 2 to 24mM copper sulfate, as measured by the minimum inhibitory concentration (MIC) necessary to inhibit bacterial growth. Somewhat disturbingly, *Salmonella* isolates were less susceptible to copper sulfate, and were inhibited at MICs of 20 to 28mM copper sulfate. If the results from the *in vitro* experiment simulate what occurs in the gut, *Salmonella* would have an advantage over other bacterial populations within the gut, particularly when these compounds are used in feed. Lastly, the enterococci populations displayed a bimodal response to the copper sulfate, suggesting two populations; one population that has not acquired copper resistance mechanisms, and a population that has acquired those mechanisms (Aarestrup and Hasman, 2004). Based on these

results, however, the authors concluded that copper resistance is currently only developed to a limited degree in intestinal bacteria.

Copper resistance is not a new phenomenon, and has developed in pathogens as a means of contending with the high levels of copper in various situations, such as high levels of copper that are fed in livestock diets. For example, in 1993, Williams et al. identified copper resistant strains of *E. coli* and *Salmonella* spp. in swine barns in the UK and Australia. Copper resistance has been investigated; Tetaz and Luke (1983) noted plasmid-controlled resistance to copper in *E. coli*, and numerous studies have confirmed these results in *E. coli* and other bacteria. In a study of *Enterococcus faecium* isolates, Hasman and Aarestrup (2002) discovered that the genes encoding copper resistance are transferable between different bacterial isolates and, in some isolates, also correlate with increased resistance to antibiotics such as macrolides and glycopeptides. Interestingly, the authors also found correlations between dietary copper concentrations and copper resistance. In Danish pigs, which are commonly fed diets with copper concentrations ranging from 25 to 165ppm, 44 of the 59 isolates (75%) were copper resistant. The authors also identified copper resistance in 10 of 29 isolates (34%) in Danish chickens, which are typically fed dietary copper concentrations of 20ppm. Pathogen resistance has not been well characterized as of yet.

In fact, only a few studies have also examined the effect of copper on pathogens such as *Salmonella* and *Campylobacter*, which are commonly associated with poultry. Faundez et al. (2004) determined that when coated with  $10^6$  CFU/ml of *Salmonella enterica* or *Campylobacter jejuni*, copper surfaces reduced the concentrations of both bacterial species from  $10^6$  CFU/ml to  $10^2$  CFU/ml within 4 h of exposure to the copper coated surfaces, but no such reduction was observed on steel or polymer surfaces. Another study, by Beal and coworkers (2003), determined that the addition of 50 ppm copper sulfate to liquid pig feed and skim milk did not significantly affect *Salmonella typhimurium* DT104:30 populations of the solutions. However, the addition of lactic acid and 50ppm of copper caused a 10-fold decrease in *Salmonella typhimurium* DT104:30 populations of the solutions, suggesting that acid stress causes reduced copper tolerance in *Salmonella typhimurium* DT104:30. Other research on acid-stressed *E. coli* has obtained similar results (Maule and Keevil, 2001).

Given the interaction between copper and bacteria, it is quite possible that pharmacological doses of copper do promote growth by inhibiting certain populations of bacteria. Unfortunately, our understanding of this mechanism is still in its infancy, and further research is necessary to elucidate the mechanisms behind copper-induced growth promotion.

## **Ionic Compounds**

Another alternative to antibiotics that has been of recent interest is sodium chlorate and ionic compounds containing sodium chlorate. When fed via oral gavage in low doses (100-200 mM) to pigs and cows, sodium chlorate selectively kills facultative anaerobes such as *Escherichia coli* O157:H7 and *Salmonella typhimurium* DT104 (Anderson et al., 2001a; Anderson et al., 2001b). These pathogens are classified as facultative anaerobes and they possess respiratory nitrate reductase which reduces nitrate to nitrite. Additionally, respiratory nitrate reductase also reduces chlorate to chlorite, a compound which is cytotoxic to the bacteria (Stewart, 1988). Since most potentially beneficial gastrointestinal microbes are not facultative anaerobes, they do not possess the respiratory nitrate reductase and are therefore not affected by sodium chlorate.

More recently, mixtures of ionic compounds have been evaluated, and these mixtures typically include oxyhalogenic compounds attached to a sodium group, such as: sodium chloride, sodium chlorate, sodium chlorite, and sodium bromide, as well as other salts such as sodium sulfate, trisodium phosphate, sodium borate, and sodium salicylate. Interestingly, with the exception of sodium chlorate, little is known about the mechanism of action in the gastrointestinal tract of the bird. A commercial mixture of oxy-halogens known as Bioxy® (pat. 4,880,638; Bioxy®, Inc., 1989; Raleigh, NC) is described as a “series of synergistic combinations of chlorine materials that are effective as microbicides, virocidic, and sporocides without utilizing chlorine dioxide itself” (Gordon, 1989). When administered in low doses (0.05 g/L) through treated water to *Salmonella typhimurium*-challenged broiler chicks, Bioxy® has been shown to improve growth, feed utilization, nitrogen retention, and to decrease cecal bacterial counts by one log, although this reduction was not significant (Pardue and Jones, 1993).

### **Bacterial Response to Stress Hormones**

Given that bacteria are so adaptive to various situations, it is not surprising that many bacteria are able to respond to stress-induced alterations within the host. Recent studies dedicated to learning the roles of stress hormones and bacterial growth have indicated that catecholamines stimulate growth of many gram negative bacterial species. To date, only one study has documented the effects of norepinephrine on indigenous bacteria of the intestine. Lyte and Bailey (1997) observed increases of 3 to 5 logs of total gram-negative bacterial populations, most notably *E. coli*, of the cecal digesta and cecal wall of mice exposed to intestinal release of NE. Many other studies have examined the effects of catecholamines on pathogenic bacteria. Lyte and Ernst (1992) were the first to determine that the growth of *Escherichia coli*, *Yersinia enterocolitica*, and *Pseudomonas aeruginosa* was enhanced significantly by NE while epinephrine also stimulated growth. Growth of each of these species was dependent upon specie, strain, and type of catecholamine. Further investigation by Lyte and Ernst (1993) determined that the growth was not mediated by either  $\alpha$ - or  $\beta$ -adrenergic receptors on the bacteria. Although the mechanism(s) that allow for the increased growth of the bacteria has not been fully elucidated to date, other research has indicated that NE increases the *in vitro* production of Shiga-like toxins, increases the expression of K99 pilus adhesion, and secretes a heat-stable autoinducer into the growth medium that stimulates growth in *E. coli* (Lyte et al., 1996a,b; Lyte et al., 1997).

Coulanges et al. (1997) has proposed that some of the effects of NE on bacterial growth are mediated by siderophore-like activity of the catecholamines. In 2000, Freestone et al. reported evidence supporting this theory and reported that NE stimulates bacterial iron uptake in the presence of lactoferrin and transferrin, agents which restrict free iron in the media.

Catecholamine-enhanced growth varies greatly among bacterial species and strains. Freestone (1999) reported that gram negative bacteria such as *S. enteritidis* responded to NE, with increases from an initial 5.9 cfu/ml to 8.0 cfu/ml when exposed to NE. Furthermore, although each of the seventeen gram negative bacteria tested responded with increased growth when cultured with NE, the degree of the responses were variable. Also tested in this experiment were six gram positive species, which responded variably to NE. Belay et al. (2003) reported that growth of *Salmonella choleraesuis* was not affected by NE, indicating strain differences within species. Lastly, Rahman et al. (2000) observed a ten-fold increase in the growth of *Salmonella*

*typhimurium* when cultured with NE and epinephrine, and increases in enterotoxin production increased roughly six-fold in media supplemented with NE

Once that research demonstrated definitive *in vitro* interactions between bacteria and catecholamines, it became important to establish that catecholamines are released into the GIT during stressful situations. The intestinal tract is richly innervated, and Costa and Brookes (1994) estimate that the number of neurons found within the GIT equal the amount found in the central nervous system. Research has indicated that a sizeable proportion of these neurons are noradrenergic, or nerves that release NE and epinephrine, and the nerve fibers extend throughout all the layers of the intestine (Furness and Costa, 1980). Because technical problems impede absolute quantification of the amount of NE present in the small intestine, actual concentrations of NE within the lumen of the intestine has not been quantified. However, because research has identified the mesenteric organs as one of the major production and deactivation sites of NE, and because of the concentration gradient present within the mesenteric organs, the spillover of NE into the intestinal lumen is likely (Lyte and Bailey, 1997), and there is enough evidence to support that theory. For instance, Furness and Costa (1987) indicated that catecholamines are released into the lumen of the digestive tract, and research by Dib et al. (1990) has indicated that emotional distress causes the release of catecholamines into the small intestine.

## **Conclusions**

Molecular tools that are currently available and that are being developed are vastly improving our knowledge of the horizontal and vertical microbial communities in the GIT of our livestock and poultry specie. We have only begun to scratch the surface on cross-talk amongst bacteria, bacterial modulin signaling to the enterocyte, commensal relationships with the mucosal epithelia, as well as conditions that perpetuate or refute induction of virulence in different bacterial specie.

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