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Gut Development and Health in the Absence of Antibiotic Growth Promoters*

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ABSTRACT : Acceptance of antibiotic growth promoters (AGP) in agricultural animal production is rapidly disappearing. Both government regulations and consumer preference are driving this change. Producers in any country that seek export markets will be forced to give up AGP if they are to sell to the EU and many other markets. This report will first review the history of AGP use in the animal industry and the concerns about development of antimicrobial resistance. A description of the development and structure of the gut and how it is affected by AGP administration will conclude with results of studies to replace AGP with antimicrobial organic acids.
(**Key Words :** Antibiotic Growth Promoter, Poultry, Swine, Organic Acids)

INTRODUCTION

Antibiotic growth promotion in agricultural animal production has been practiced for about 50 years in the United States and other countries. Early indications of a beneficial effect on production efficiency in poultry and swine were reported by Moore et al. (1946) and by Jukes et al. (1950). One of the first reports of resistance in food animals was made by Starr and Reynolds (1951) after experimental feeding of streptomycin in turkeys. Other researchers reported an association of resistance to tetracycline with the feeding of growth promoting levels of the antibiotic to chickens (Barnes, 1958; Elliott and Barnes, 1959). Early concern about the development of antibiotic resistance in human pathogens and recommendations to ban sub-therapeutic use in animal feeds was discussed by Swann in a report to the British Parliament (1969). Indeed, evidence exists that antibiotic resistance genes can be and are transmitted from animal to human microbiota (Greko, 2001). Monitoring and identifying resistance mechanisms and their dissemination into the food chain were reviewed by Roe and Pillai (2003). Pathogenic bacteria resistant to a number of antimicrobial agents emerged worldwide in the 1980's (Aarestrup, 2003). As these were detected, several reports were published recommending a ban on antimicrobial use in food animals as a precautionary measure.

The World Health Organization (WHO) published a report on the medical impact of the use of antimicrobials in food animals suggesting a link between agricultural use of AGP and increasing infections of humans by antibiotic resistant microorganisms on an epidemiological basis (1997). WHO has issued a report entitled Global Principles for the Containment of Antimicrobial Resistance in Animals Intended for Food (World Health Organization, 2000). This report recommends, on precautionary grounds, that national governments adopt a proactive approach to reduce the need for antimicrobial use in animals and establish surveillance of antimicrobial usage and resistance. With respect to the use of antimicrobial growth promoters, WHO suggests that use of antimicrobial growth promoters that are in classes also used in humans be terminated or rapidly phased out, by legislation if necessary, unless and until risk assessments are carried out (World Health Organization, 2000). The organization also suggests that animal health management should be routinely practiced so as to avoid the prophylactic use of antimicrobials and antimicrobial availability should be limited to therapeutic use by prescription. The recommendations are precautionary, based on the potential for a reservoir in food animals of an antibiotic resistant bacterial population (primarily enterococci) that could be transferred to humans.

On a global level, a joint workshop was held involving

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the WHO, the Food and Agriculture Organization of the United Nations (FAO) and the World Organization for Animal Health (OIE) on non-human antimicrobial usage and antimicrobial resistance (Geneva, December 2003). The resulting report recommends implementation of the WHO global principles for the containment of antimicrobial resistance in animals intended for food (World Health Organization, 2004) as noted above. In addition, the report recommends the implementation on a national level of risk assessment studies and establishment of surveillance programs to monitor AGP use and antimicrobial resistance in bacteria from food animals (World Health Organization, 2004). The use of risk assessment models in evaluating and regulating food animal antibiotics has recently been reviewed (Cox, 2004).

In February 1998, Danish cattle and chicken producers voluntarily stopped use of all antimicrobial growth promoters as did producers of swine for finisher pigs (World Health Organization, 2003). In July and September 1999, other individual growth promoters were banned by the EU Commission because they belonged to classes of antimicrobials also used in humans (tylosin, spiramycin, bacitracin, and virginiamycin), or were considered unacceptable occupational toxicity risks (olaquindox and carbadox). In December 1999, the Danish swine industry voluntarily stopped the use of all remaining antimicrobial growth promoters in swine under 35 kg (World Health Organization, 2003). Thus, Denmark has restricted the use of antimicrobials to therapeutic use, by prescription only, since January 2000. Use of some anticoccidials in the poultry industry is still permitted.

There is some information on the consequences of removing AGP from poultry and swine production in Denmark. For the broiler industry, productivity (kg broilers produced/m2/growout) has not been affected by the ban of AGP, nor has livability (Emborg et al., 2002). Feed conversion, however (total kg feed used per grow out/total kg live weight per grow out), did increase by 0.016 kg/kg from November 1995 to May 1999 (1.78 to 1.796). There was very little additional change from 1999 to June 2002 (Emborg et al., 2002). It should be noted that feed efficiency went to highs of 1.83 immediately after the ban and to more than 1.84 in late 1999 (Emborg et al., 2002).

The major health concern in poultry, necrotic enteritis (NE), did not increase enough after the AGP ban to give an increase in mortality. It should be noted, however, that the consumption of the ionophore coccidiostat salinomycin, which has activity against Clostridium perfringens (Watkins et al., 1997; Elwinger et al., 1998; Martel et al., 2004), has increased steadily in Denmark since the ban on AGP. Consumption of salinomycin in 1996 was 4,500 kg (active compound) and 11,213 kg in 2002 (DANMAP, 2002). This may, in part, explain the successful control of NE since the AGP ban in Denmark in 1999.

Finally, these relatively positive health and productivity results for poultry are in contrast to those for swine, where withdrawal of AGP in weaner pigs was associated with a decline in average daily gain from 422 g in 1995 to 415 g/day in 2001 and an increase in mortality over the same period from 2.7% to 3.5% (Callesen, 2003).

The reality that AGP use is being curtailed by market, if not legislative, actions has led to a new urgency in the search for replacements. Following early demonstrations that oral antibiotics do not have growth promoting effects in germ-free animals (Coates et al., 1955; Coates et al., 1963) and the observation that some of these antibiotics are not absorbed, studies of the mechanism for growth promotion have focused on interactions between the antibiotic and the gut, including its commensal microbiota. As a part of the search for AGP replacements, a review of the development and structure of the gut and its associated mucosal immune system can provide a foundation for understanding the effects of AGP on this system.

GUT AND IMMUNE DEVELOPMENT

Prenatal

Developmentally, the GI tract is derived from endoderm surrounded by splanchnic mesoderm and can be distinguished into foregut, midgut and hindgut by day 3 of incubation (Gilbert, 1997). The endoderm will give rise to the epithelial lining of the GI tract and the ducts of the mucous glands, while the mesoderm will give rise to the muscular wall and connective tissue. The primitive gut tube is the source of a number of organs, many of which are members of the digestive system. For example, the liver, pancreas and gall bladder all arise from the embryonic gut tube (Romanoff, 1960). By day 6, the future intestine can be recognized to consist of duodenal loop, small intestine and cecum. The primitive midgut is open and is connected to the yolk via the yolk duct, so the walls of the yolk sac and the walls of the GI tract are continuous (Freeman and Vince, 1974). The primitive hindgut will give rise to the cloaca and bursa. The Bursa of Fabricius forms as a diverticulum on the dorsal side of the cloaca by day 4 (Romanoff, 1960), and is invaded by lymphocytes on days 12 and 13 (Grossi et al., 1977). These cells are committed B-cells, but are only capable of IgM expression at hatch (Leslie and Martin, 1973).

Postnatal

The general direction of development is anterior to posterior, with the foregut being the most differentiated at the time of hatch (Romanoff, 1960). Although the bird has not ingested feed at the time of hatch, intestinal and pancreatic enzymes as well as nutrient transport capabilities are present (Buddington, 1992). Nutrient digestibility,

however, is not fully mature at this time (Krogdahl and Sell, 1989). Ontogenetic changes, which accompany improved digestion, include increased levels of pancreatic and intestinal enzymes (Sell et al., 1991; Noy and Sklan, 1995; Uni et al., 1998, 1999), increases in overall GI tract surface area for absorption (Nitsan et al., 1991), and changes in nutrient transporters (Buddington and Diamond, 1989). Hatchling poultry undergo a fundamental change in nutrient supply and metabolism upon emergence from the egg shell. During embryonic incubation, yolk lipid supplies the caloric needs of the bird and is delivered from the yolk sac via the blood stream. As hatch approaches, glycogen accumulates in the liver and is used during the hatching process (Freeman and Vince, 1974). In addition, lipid is transferred to the liver of the embryo and the yolk sac is internalized within the body wall (Romanoff, 1960). If the animal is denied food and water, this lipid and the residual yolk water and protein can supply nutrients to the hatchling until feed and water become available; however, the residual yolk nutrients are more valuable for their ability to confer passive immunity and as structural material for the developing bird (Dibner et al., 1998). Evidence confirms that some of the residual yolk makes its way into the intestine via the yolk stalk and, thus, provides digestible nutrients that may stimulate maturation of the digestive and absorptive functions of the neonatal intestine (Noy et al., 1996; Sulaiman et al., 1996).

The immune system of the bird is partly developed at hatch. The primary immune organs, the thymus and bursa, are both present and populated by lymphoid cells. The migration of lymphocytes to the thymus occurs in several waves, beginning at day 6 of embryogenesis. These cells pass through the thymus and populate peripheral tissues (Bucy et al., 1988). The thymocytes are $CD3^+$ (avian homologue), with the γ/δ positive thymocytes arriving before the α/β positive thymocytes in each successive wave (Dunon et al., 1997; Bar-Shira et al., 2003). The γ/δ positive thymocytes reside mainly in the intraepithelial location while the α/β positive thymocytes are mainly found in the lamina propria (Bucy et al., 1988; Bar-Shira and Friedman, 2005). In peripheral organs such as the intestine, development of T-cell effector function occurs primarily after hatch (Jeurissen et al., 1989). Cells in the thymus develop CD4 or CD8 antigens during embryogenesis and there are some CD4+ and CD8+ thymocytes in the intestinal epithelium at one week of age (Lillehoj and Chung, 1992). Among the intraepithelial lymphocytes, the percent of CD8+ T-cells increases with age while CD4+ cells decline (Lillehoj and Chung, 1992).

Humoral immunity follows much the same pattern. At hatch, mRNA expression of the Bu-1 antigen, which is expressed on B lymphocytes throughout their development, was higher in the cecal tonsils compared to other parts of the intestine but increased with age in all parts of the intestine (Bar-Shira et al., 2003). Ontogenetic studies of lymphocyte distribution indicate that interferon and interleukin-2 mRNA increase after post-hatch day 4 (Bar-Shira et al., 2003). The ability to mount a secondary response, as indicated by the presence of germinal centers or circulating IgG and IgA, begins to appear between one and four weeks of post-hatch life in the broiler chick (Leslie, 1975; Mast and Goddeeris, 1999; Bar-Shira et al., 2003).

Recent research in mice has shed light on the interaction of the gut associated immune system and the resident microflora. Macpherson has studied the role of dendritic cells in the development of secretory IgA in the GI tract (Macpherson and Uhr, 2004). The induction of commensal-specific IgA was found to involve the capture of live bacteria by the dendritic cells in the intestinal lamina propria. This involves the prying open of enterocyte tight junctions and the sampling of microbial antigens by dendritic cell extensions (Rescigno et al., 2001). Some of the bacteria survive in the dendritic cells and are carried into the mesenteric lymph nodes (Macpherson and Uhr, 2004). This appears to stimulate the production of IgA specific to the commensal bacteria using a mechanism independent of T-cells (Macpherson et al., 2000). The B lymphocyte secreting this IgA may arise from an IgA+ lymphoblast that has homed to the GI tract lamina propria (Macpherson et al., 2000) or from IgM+ B cells that can switch to production of the IgA isotype without T cell help (Fagarasan et al., 2001). Either way, this would provide a mechanism for the response of the GI tract to commensal bacteria without the concomitant burden of an inappropriate systemic immune response. These findings remain to be tested in birds.

Gut immune cells combat not only pathogenic bacteria and their toxins, but also the overgrowth of or inappropriate attachment by the normal microflora. Evidence here is from studies of germ-free animals, which exhibit delayed lymphocyte and other immune cell development in the lamina propria and far fewer IgA-producing cells when compared to conventionally-reared animals (Gordon and Pesti, 1971; Berg and Savage, 1975; Umesaki et al., 1993; Rothkotter et al., 1994; Umesaki et al., 1999). For example, the development of antibody diversity in poultry is inhibited by germ-free growth conditions (Schaffner et al., 1974; Ekino et al., 1980). Indeed, the majority of evidence supports the notion that the intestinal immune system develops in parallel with the development of the normal microflora. Introduction of even a single species of commensal bacteria into germ-free animals can stimulate the development of the secretory IgA system (McCracken and Gaskins, 1999).

It should be noted, however, that while the microflora-induced development of the intestinal immune system may be key to the long term health of the animal, there is inherent inefficiency when immune stimulation is maintained at a chronic level as appears to be the case in conventional vs. germ-free animals (Gordon et al., 1963). The typical adult human, for example, secretes more than 5 grams of IgA each day, much of which is specific to antigens from the resident microflora (Macpherson et al., 2000). Thus, microflora-specific IgA secretion alone can cost the animal several hundred grams of protein over a lifetime that isn't directed towards growth. Macpherson et al. (2000) report that the background IgA against commensal bacterial antigens is directed toward the individual's established gut flora with a further or renewed response to antigen change within that established gut flora. Thus, reducing changes in the gut microflora by controlling species that enter from the food or the environment should reduce the additional response of the gut immune system to changes in microflora antigenic stimulation.

GUT STRUCTURE AND FUNCTION

The overall microscopic anatomy of the GI tract is quite consistent throughout its length. It is a tube whose inner lining consists of a complex and highly differentiated epithelium supported and surrounded by loose connective tissue and muscle (Andrew and Hickman, 1974). This is the mucosal layer. The epithelium differs extensively along the length of the GI tract, changing according to the digestive function of the different gut segments. The outer lining, the submucosa, primarily consists of smooth muscle in two layers, an outer longitudinal layer and an inner circular layer. Between the layers of muscle can be found the blood vessels, lymphatics and complexes of autonomic nerves (Moran, 1982). This general pattern can be seen from the esophagus to the colon. Even the cecal tonsils and bursa have this general structure, with the loose connective tissue of the mucosa completely filled with lymphocytes.

Differentiation of the epithelial cells along the length of the GI tract continues to be a source of interesting information about the development and function of the digestive system. The epithelium varies from a squamous lining in the esophagus and crop to the highly specialized acid-secreting cells of the proventriculus. In the small intestine, the epithelium is thrown into long folds, the villi, which serve to increase the surface area for enzyme secretion and nutrient absorption. The epithelium on the villi is found in a single layer of columnar cells, which are specialized for mucous secretion (goblet cells), nutrient absorption (absorptive enterocytes), or hormone-secretion (enteroendocrine cells). The apical membrane of the absorptive enterocytes is further increased by the formation of microvilli. When healthy, this villus epithelium is impermeable to macromolecules and microbes by virtue of the lateral tight junctions that fuse the cells together. Intra-epithelial lymphocytes, including antibody-secreting plasma cells, are found in the lamina propria, and anti-microbial peptide-secreting Paneth cells are found between the villi in deep structures called crypts.

The intestinal epithelium is a renewing cell population. Like Paneth cells, stem cells are located in the crypts. Most of the enterocyte cell division takes place in the crypts and is followed by a sliding migration of the cells up the villus. However, in contrast to mammals, proliferation of enterocytes in the chick jejunum also occurs along the villus (Uni et al., 1998). The cells undergo differentiation as they migrate, becoming fully functional absorptive enterocytes, goblet cells or enteroendocrine cells. These changes are both structural and functional, as described below. As the cells reach the villus tip, they undergo apoptosis and are shed into the intestinal lumen. The extrusion zone, where this shedding takes place, is easily visualized by scanning electron microscopy (Bayer et al., 1975). This is a highly coordinated phenomenon, with sloughing of the dead cells being balanced by the replacement of immature cells from the mitotic activity of the stem cell population in the crypts. This epithelium renews itself faster than any other tissue in the body, replacing itself in as little as two days (Imondi and Bird, 1966).

Goblet cells (named after their shape) are highly polarized, short lived, secretory cells with their apical membrane facing the intestinal lumen. These cells are specialized to secrete a mixture of glycoproteins called mucins, which are the primary component of gastrointestinal mucus (Quigley, 2001; Van dijk et al., 2002). Goblet cells also secrete a variety of metal cations, including iron, zinc, lead and calcium (Bauman et al., 1987), and lectins (Beyer and Barondes, 1982). Mucus lubricates the lining of the GI tract, protecting it from mechanical injury, stomach acid, and pathogenic microbes and viruses. Diet composition can affect the relative number of goblet cells, and the chemical composition of the secreted mucins (Sharma et al., 1997; Langhout et al., 1999; Fernandez et al., 2000; Smits et al., 2000). In turn, these differences in mucins can alter the susceptibility or resistance to colonization by pathogens (Fernandez et al., 2000). Defense against pathogens is also provided by a group of proteins in the mucus called defensins, which are secreted by the Paneth cells. Defensins are short polypeptides, usually 12-50 amino acids which insert themselves into the cell membrane of a variety of microorganisms creating holes. Defensins kill a broad variety of microbes including both Gram-negative and Gram-positive bacteria, yeast and other fungi, protozoa, nematodes, and enveloped viruses (Bals, 2000; Alberts et al., 2002). Still, the resident microflora

necessitates great increases in mucus secretion and GI tract epithelial cell turnover. Because many bacterial species enzymatically digest away the mucus layer, the host must constantly secrete more (Gaskins, 2001).

Absorptive enterocytes, or brush-border cells, are columnar cells that exhibit an array of fingerlike protrusions, called microvilli, on their apical surface. These microvilli are supported by bundles of actin, and provide a 30-fold increase in the surface area of absorptive membrane (Bals, 2000). This increased surface area allows for greater absorption of nutrients, and provides a point of anchoring for a variety of enzymes involved in extracellular digestion of nutrients. The mechanism of nutrient absorption depends on the particular molecule being taken up. Uptake can occur by diffusion, or via a wide variety of sugar, amino acid, fatty acid and other molecular transporters found in the enterocyte plasma membrane (Hill, 1979).

Effects of antibiotic growth promotants on the gut

Orally ingested antibiotics promote growth and efficiency of poultry and other animals. The effect can include gain but often is limited to feed efficiency effects only. Thus, direct effects of AGP on the microflora can be used to explain decreased competition for nutrients, and reduction in microbial metabolites that depress growth (Visek, 1978a; Anderson et al., 1999). Additional AGP effects that also occur in germ-free animals include reduction in gut size, including thinner intestinal villi and total gut wall (Coates et al., 1955). The may be due, in part, to the reduction of trophic luminal short chain fatty acids that are product of microbial fermentation (Frankel et al., 1994) The reduction in gut wall and villus lamina propria have been used to explain the enhanced nutrient digestibility seen with AGP (Jukes et al., 1956; Franti et al., 1972; Anderson et al., 1999).

One recent experiment to investigate the effect of antibiotics on the microflora was performed by Gaskins and colleagues (Collier et al., 2003). In this paper, 4 week old barrows were ileally-cannulated and placed on a no antibiotic diet, or diets with tylosin or a weekly rotation of antibiotics. The effects of the different treatments were analyzed using two methods from molecular biology: PCR-DGGE (polymerase chain reaction, followed by denaturing gradient gel electrophoreses) and quantitative PCR. The results of these experiments were that, relative to the non-antibiotic-treated controls, antibiotic treatments reduced both the species diversity and the total numbers of bacteria, including *Lactobacilli* (Gaskins et al., 2002).

Finally, the reduction in opportunistic pathogens and subclinical infection has also been linked to use of AGP. It should be noted that injection of bacterial metabolites such as lipopolysaccharide or immune mediators such as interleukin-1 can mimic the reduced efficiency of an animal with a conventional microflora and no antimicrobial in the diet (Roura et al., 1992), and this illustrates the importance of the host response to the microflora as another factor limiting growth efficiency. The reduction in microflora, and its consequences, may be the underlying mechanism for beneficial effects of antibiotics. It has been proposed that the reduction in amino acid catabolites, the prevention of bile catabolism, and the reduction in chronic immune stimulation are among the primary mechanisms by which antibiotics improve animal performance (De Somer et al., 1963; Visek, 1978a, b; Feighner and Dashkevicz, 1987, 1988; Gaskins et al., 2002).

Among the candidates for AGP replacement, organic acids appear to have the most widespread acceptance at this time (Dibner and Buttin, 2002; Dibner, 2003). A recent study examined the effect of feeding antimicrobial organic acids on the performance of birds subjected to a combined coccidial (day 14) and clostridial (days 18, 19 and 20) challenge (Hofacre, 2005). In this experiment, the treatments consisted of unchallenged birds, challenged birds, birds challenged but treated with an AGP (BMD), and birds challenged but treated with an organic acid blend. In this study, post challenge performance for the non-challenged birds was significantly better than for any of the challenge treatments. Among the challenged treatments, the organic acid blend gave performance significantly better than the untreated birds and numerically superior to the antibiotic treatment. Thus, organic acids can be an important contributor in a strategy to reduce use of AGP for high-efficiency animal agriculture.

CONCLUSIONS

It seems inevitable that the use of AGP will decline in the future. Where legislation is not already in place, consumer pressure is building to make the practice of using antimicrobials economically impractical because of market limitations and export restrictions. Use of germ-free animals to model the effects of AGP suggests that most of the benefits of antimicrobials derive from effects on the intestinal microflora. Antimicrobial organic acids appear to provide benefits similar to AGP. More research is required to determine whether the mechanism of action of organic acids is the same as AGP and whether other measures must be taken to restore full AGP-like improvements in efficiency. Nevertheless, organic acids provide a natural alternative to AGP that can be put in to use today.

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