

*Thirty-Second Annual*  
**CAROLINA  
POULTRY NUTRITION  
CONFERENCE**

*OCTOBER 26-27, 2005*



**Sheraton Imperial Hotel  
I-40 Exit 282 at Page Road  
Research Triangle Park, North Carolina 27709  
(919) 941-5050**

**Sponsored by  
Carolina Feed Industry Association  
in cooperation with  
NC State University  
and  
NC Department of Agriculture & Consumer Services**

**POULTRY NUTRITION CONFERENCE – Auditorium  
THURSDAY, OCTOBER 27**

7:00 a.m. **Registration** - Auditorium Foyer

**Session I**

**Vernon Felts – Session Chairman**

8:00 a.m. **Welcome** – Jim Stevens, CFIA President

8:15 a.m. **Dietary factors that affect gut health and pathogen colonization**  
– Peter R. Ferket, North Carolina State University.

9:00 a.m. **Nutrients, nutritional states and small intestinal microbiota**  
- Todd Applegate, Perdue University.

9:45 a.m. **Coffee Break**

**Session II**

**Scott Crow – Session Chairman**

10:15 a.m. **Assessing the results of European union ban on antibiotic feed additives** - Hector Cerventes, Phibro Animal Health

11:00 a.m. **Impact of a ban on animal by-products and antibiotic growth promoters – European Experience**  
- Peter Woodward, Nicholas Turkey Breeding Farms

11:45 a.m. **Luncheon**

**Session III**

**Julian Brake – Session Chairman**

1:30 p.m. **Nutritional factors that affect leg problems in meat poultry: a review**  
- Edgar Oviedo, North Carolina State University

2:15 p.m. **Overview of the immune dynamics of the digestive system**  
- Doug Korver, University of Alberta

3:00 p.m. **Coffee Break**

**Session IV**

**Bob Buresh – Session Chairman**

3:30 p.m. **Organic versus inorganic trace minerals in poultry: the zinc experience**  
- Thim Cheng, Zinpro Corporation

4:15 p.m. **Use of biotechnology in nutrition research**  
- Chris Ashwell, North Carolina State University

5:00 p.m. **Adjourn**

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# **Dietary Factors that Affect Gut Health and Pathogen Colonization**

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The poultry industry has undergone remarkable change and growth over the last 30 years, and it will continue to expand in the coming years to meet higher demand for low-cost, healthy and convenient products. In addition to the expanding market, commercial poultry is being genetically selected for ever increasing growth performance and efficiency. This selection for increased growth rate has resulted in changes in gastrointestinal development during growth of the animal (Tottori *et al.*, 1997). Apparently, young poultry are more susceptible to enteric pathogens today than they were 30 years ago. These pathogens have been of concern to the poultry industry because of lost productivity, increased mortality, and contamination of poultry products for human consumption (Patterson and Burkholder, 2003).

Prophylactic and therapeutic uses of antibiotics have been the main tools used by the poultry industry to prevent or to treat disease due to enteric pathogens. However, the voluntary or legislated limits on the use of antibacterial feed additives for poultry is motivating poultry scientists to better understand the role of commensal gut microflora and how it can be modified or stabilized by various “natural” alternatives to antibiotics. Some of these natural alternatives include prebiotics, probiotics, enzymes, acidifiers, herbs, essential oils, and immunomodulators. Many enteric pathogens do not develop mechanisms of resistance to these natural dietary components and they have been shown to shift the intestinal microflora to more stable and healthier state (Lan *et al.*, 2005). Understanding the role of gut microorganisms in nutrition and health is vital for achieving future sustainability and for improving the efficiency and environmental acceptability of poultry production. The objective of this paper is to review nutritional strategies to modulate gut health and control pathogen colonization with emphasis on potential natural alternatives to antibiotics.

## **Intestinal Tract Ecosystem**

The major purpose of the digestive system is to assimilate nutrients required for energy, maintenance, growth, and reproduction. Digestion consists of a number of physical and chemical processes. Feed is ingested, broken down into smaller particles, macerated, mixed with digestive enzymes, and propelled through the digestive tract by the muscular activities of the tract. Salivary, gastric, pancreatic, biliary, and intestinal secretions collectively provide enzymes that aid in digestion, watery medium, and optimal pH required for digestion. Digestive enzymes hydrolyze carbohydrates, protein, and lipids into a limited number of much smaller compounds suitable for absorption. Mucus, secreted onto the mucosal lining, protects and lubricates the gastrointestinal tract. Microorganisms, indigenous to the digestive tract, can provide additional nutrients by breaking down structural carbohydrates that are not subject to digestion by endogenous enzymes and by synthesizing amino acids and vitamins essential to the host animal.

The gastrointestinal tract the main portal through which pathogens invade the body and cause disease; but, the animal has natural protective barriers to prevent this invasion. Indeed, there are more bacterial cells in the digestive tract of most animals than there are cells of the animal itself.

Pathogenic bacteria and viruses are discouraged from entering the body by physical barriers such as skin, intestinal mucosa, and intestinal flora. If pathogens breach these barriers, the immune system and its associated defenses mechanisms spring into action in order to minimize systemic infection. These natural defenses rely on precise physiological mechanisms that are genetically predetermined. However, the expression and efficacy of these mechanisms is dependent upon the presence of specific external elements, including dietary nutrients that meet the metabolic demands for maintenance and growth.

The intestinal ecosystem contains a very diverse community of microbial cells that influence the host in various ways. According to Lan et al. (2005), some intestinal microflora have beneficial effects on the host, such as: 1) promotion of gut maturation; 2) enhancement of gut integrity; 3) antagonisms against pathogens (competitive exclusion); and 4) immune modulation. The microflora also plays a significant role in maintaining intestinal immune homeostasis and prevent inflammation. The strongest determinant of the gut microbial ecosystem is the diet of the host. Apajalahti *et al.* (2001) surveyed the microbial community of broilers raised at eight commercial poultry farms in Finland and fed different commercial wheat-based diets, some with locally added whole wheat. This survey covered different seasons (spring and fall) and years (1997, 1998, and 2000). They found that diet was the strongest individual determinant of the total microbial community structure in the ceca of broiler chickens, whereas profiles of individual farms with identical feed regimes hardly differed from each other. There was also no significant variation of the colonic microbial community due to season or year. Therefore, it should be possible to shift the microbial community from pathogenic to beneficial bacteria by changing the dietary composition of ingredient (Gibson and Roberfroid, 1995; Collins and Gibson, 1999).

The balance in this ecosystem is also imposed by the animal host responses to control the bacterial proliferation in their intestines using several different physiological mechanisms. These mechanisms include chemical processes (e.g. acid and bile production), highly competitive rate of nutrient absorption, high passage rates of digesta that washes out free bacteria, continuous sloughing of the epithelial cells and mucus that washes out adhered bacteria, and secretion of immunoglobulin A. Any unbalance caused by changes in general immunity of the bird, temperature stress (heat or cold), diet composition, or any other inefficiency of the host to maintain low numbers of bacteria can cause a disbacteriosis and/or enteritis with lower absorption of nutrients by the host.

There are multiple causes for secondary enteritis. Some of the most common reasons are bad litter quality, poor hygiene and ventilation, and low quality of feed or high variability in its composition. Any of these factors or a combination of several of them can trigger a gut health problem. Coccidiosis is a common parasitism in poultry and one of the more common causes of enteric problems. *Coccidia* infection causes reduced weight gain and poor feed conversion efficiency, reduced feed and water intake, increased intestinal passage time, decreased digesta viscosity and nutrient digestion, villous atrophy, intestinal leakage of plasma proteins, and increased intestinal acidity (Williams, 2005).

Coccidial infection has been shown to increase broiler chicken susceptibility to necrotic enteritis, which suggests that the parasite changes the rules of bacterial competition in the gastrointestinal tract for the advantage of *Clostridium perfringens* (*Cl. perfringens*) (Van Immerseel et al., 2004; Williams, 2005). Apajalahti (2004) presented data that indicates that *Eimeria maxima* infection changes microbial fermentation in the gastrointestinal tract of broilers. Presumably, the damage to the mucosa following *Eimeria* challenge is incremental with time until the immune system

deals with the pathogen or the damage to the intestinal wall has been healed. Initial changes in gut environment chemistry would therefore be limited to the site of infection in the small intestine. As the *Eimeria* challenge reaches peak, sloughed cells and undigested feed would reach the caeca, which may explain why the short chain fatty acid levels in the caeca of the challenged and unchallenged birds appear to diverge after those of the ileum. Apalajahti (2004) suggested that under commercial conditions, the changes in MC caused by *E. maxima* infection are continuous and less acute, since oocyst recirculation and the host immune and physiological responses affect the gut environment. He also presented data that showed that MC shifts caused by *E. maxima* challenge are temporary. Oviedo et al.(2005a, b, c, d) have observed in several studies that vaccination against coccidia with viable oocysts by itself causes small changes on intestinal microflora, and that stresses and coccidia challenge result in drastic shifts in microbial communities that can be modulated with feed additives.

The responses to all feed additives including the growth promoting antibiotics are very variable. Extensive literature reviews (Thomke et al., 1998; Huyghebaert, 2003) have concluded that the average benefit of growth promotants is 3 to 4% with a range that goes from no benefits at all to 8% maximum.

## **Current Antibiotic Debate**

Antibiotics are natural metabolites of fungi that inhibit the growth of bacteria by altering certain properties of bacterial cellular metabolism resulting in impaired growth or death. Some antibiotics interfere with the building and maintenance of the cell wall, while others interrupt proper protein translation at the ribosomal level (Ferket, 2003). Unfortunately, the long term and extensive use of antibiotics for medical and veterinary purposes have resulted in selection of resistant bacterial strains, and genes encoding for this resistance have been also transferred to other formerly susceptible bacteria, thus posing a threat to both animal and human health (Montagne *et al.*, 2003). The unifying concept of resistance is that the microorganism under stress will sense a deteriorating environment and undergo a programmed molecular response by which specific, stress-inducible proteins are synthesized. These proteins presumably act to prevent or repair macromolecular damage caused by the stress. Some stress proteins are induced by various stress conditions, while others are induced in response to a specific stress.

Antibiotic usage by the food animal industry has come under increasing scrutiny by some scientists, consumers, and government regulators because of potential development of antibiotic-resistant bacteria, including pathogenic strains. Controlling gram-negative bacteria, like *E. coli* and *Salmonella* spp. have generated the strongest objection to antibiotic use (Gustafson and Bowen, 1997). For example, Nayak and Kenney (2002) showed that 25% of the *Salmonella* isolates from turkey flocks in West Virginia were resistant to one or more antibiotics, including gentamicin, spectinomycin, streptomycin, tetracycline, tobramycin, and sulfamethoxazole. Consequently, some countries have banned (Sweden started on January 1986) or limited (European Union started on January 2000 and total withdrawal will start on January 2006) the general use of antibiotics as growth promotants in livestock feeds. As a result of this ban on antibiotic growth promotants is a rise in the incidence of colibacillosis and necrotic enteritis caused by *Clostridium perfringens* in poultry (Truscott and Al-Sheikhly, 1997; Ferket, 2003). Prohibiting antibiotic use has caused a decrease in performance, nutrient uptake, gut health, and economical losses in livestock production. Lastly, the antibiotic ban has increased the susceptibility of foodborne pathogen colonization in the poultry intestine and contamination of poultry products for human consumption.

## Nutritional Strategies to Modulate Gut Health and Pathogen Colonization

Several strategies have been proposed as a means to manage gut health. Effective use of feed additives to manage gut health is dependent upon some degree of understanding of their mechanisms of action (Figure 1). Growth-promoting antibiotics work in part by decreasing the microbial load in the gut, resulting in a reduction in energy and protein required to maintain and nourish the intestinal tissues; thus, more nutrients are partitioning toward growth and production. In contrast, most natural feed additives do not reduce overall microbial loads. Instead, they alter the gut microflora profile by limiting the colonization of unfavorable bacteria, which promote the activity or growth of more favorable species. These natural feed additives promote gut health by several possible mechanisms: altering gut pH; maintaining protective gut mucins; selection for beneficial intestinal organisms or against pathogens; enhancing fermentation acids; enhancing nutrient uptake; and increasing the humoral immune response.

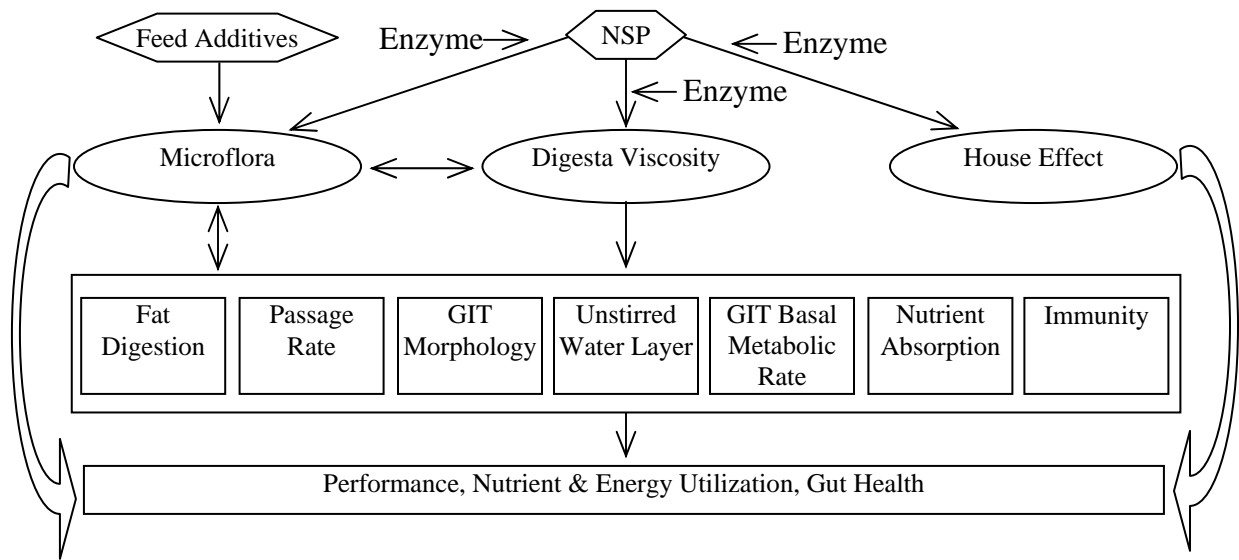


FIGURE 1: Flowchart of the modes of action of feed additives, non-starch polysaccharides, and enzyme supplementation.

## Enzyme Supplementation

Supplemental enzymes have become common additives to poultry and swine feeds to improve nutrient utilization and production performance characteristics. Enzymes are organic catalysts that initiate or accelerate reactions occurring in organic matter that would not otherwise proceed at an appreciable rate (Schaible, 1970). Even the simplest living organisms contain multiple copies of nearly a thousand different enzymes. With the development of enzyme products targeting specific substrates, the use of enzymes to improve the nutritional value of feed has received increased attention. Since the 1920s, researchers have observed beneficial effects from enzyme supplementation in poultry feeds, particularly feeds that contain grains with a high fiber component (Hastings, 1946; Moran Jr. and McGinnis, 1968; Pettersson and Aman, 1989; Santos *et al.*, 2004a). Supplemental enzymes in the feed are used to achieve one or all the following objectives: 1) increase the animal's own supply (Schaible, 1970); 2) alleviate the adverse effects

of antinutritional factors, such as arabinoxylans,  $\beta$ -glucans, etc; 3) render certain nutrients more available for absorption and enhance the energy value of feed ingredients (Classen and Bedford, 1991; Lyons, 1993); and 4) indirectly modify enteric microflora ecosystem. Consequently, enzyme supplementation improves performance parameters.

Commercial enzyme products are typically a blend of several different enzymes that are effective on a wide variety of substrates. The enzymes with proven efficacies for animal husbandry include xylanase, arabinoxylanase,  $\beta$ -glucanase, cellulase, phytase (Choct and Kocher, 2000), proteases (Odetallah *et al.*, 2003), and phospholipase (Santos *et al.*, 2004b). The following is a brief description of NSP-enzymes that has shown positive effects on gut health and pathogen control.

### ***Non-Starch Polysaccharide (NSP) Enzymes***

Today, pentosanase (NSP-enzymes) are used in virtually all poultry and swine diets comprised of mainly of small grains, such as wheat, barley, oats or triticale. Supplementing cereal-based diets with microbial enzyme preparations capable of hydrolyzing endosperm cell walls, may improve dietary nutrient availability by several means. Endoxylanase for example, degrades the xylan backbone of arabinoxylan into smaller units, which has several beneficial consequences. It renders the xylose units more available to monogastrics (Odetallah, 2000). It disrupts the water holding capacity of the NSP (Scott and Boldaji, 1997) and reduces the viscosity of the digesta in the small intestine (Bedford and Schulze, 1998; Choct *et al.*, 1999). Reduced digesta viscosity increases the diffusion rates of nutrients and endogenous enzymes enabling the bird to digest and absorb more nutrients (Pawlik *et al.*, 1990). Endoxylanase releases entrapped nutrients for the digestion by the endogenous enzymes of the bird (Chesson, 2000). Endoxylanase inhibits the proliferation of the fermentative microorganisms in the small intestine by increasing the digesta passage rate and nutrient digestion (Choct *et al.*, 1999). Thus, nutrient utilization is improved by reducing the competition between the host and its enteric microflora (Santos *et al.*, 2005).

Several studies about the effects of NSP and NSP-enzymes on bacterial community have been carried in our laboratory. We recently showed that higher concentration of plant cell polysaccharides in the diet shifted the intestinal microflora of poult to a healthier state through decreased cecal *Salmonella* spp. population, improved animal performance (Santos *et al.*, 2005) and improved gut morphology (data not published yet). In addition, we showed that turkeys fed diets high in plant cell wall polysaccharides supplemented with a blend of xylanase, protease and amylase enzymes had lower cecal *Salmonella* spp. population than turkeys on the same diet without enzyme supplementation (Santos *et al.*, 2005). NSP-enzymes hydrolyze the polysaccharides to oligosaccharides. Polysaccharides are not selectively fermented and persist through the distal colon, although symbiotic bacteria in the ceca can ferment oligosaccharides formed from the hydrolysis of NSP. In agreement with the literature, most of the commercial prebiotics have rather low molecular weight and they are generally fermented in the proximal colon (Rastall and Maitin, 2002).

Cecal symbiotic bacteria have important and specific metabolic, trophic and protective functions. Metabolic functions include fermentation of non-digestible dietary residue and endogenous mucus, which is important for the recovery of energy as short-chain fatty acids (SCFA), production of vitamin K, and absorption of ions. Symbiotic bacteria influences epithelial cell proliferation and differentiation due to their production of SCFA, and they also positively influence the development and homeostasis of the immune system. Symbiotic microflora also protects the host against pathogens through a variety of mechanisms. The attachment of non-

pathogenic bacteria to the brush border of intestinal epithelial cells can prevent the attachment and subsequent entry of pathogens. Symbiotic bacteria also competitively exclude pathogenic bacteria by competing for available nutrients, by producing bactericins, or maintaining their habitat by consuming resources of the gut and secreting compounds that are inhibitory to pathogens. Therefore, oligosaccharides formed from the breakdown of NSP molecules by enzymes discourages the colonization of enteric pathogens by increasing the resistance of the resident symbiotic microflora.

Because supplemental enzymes mediate their beneficial effects primarily by enhancing feed digestibility and nutrient availability to the host, it must be assumed that they also influence the gut microbial ecosystem by limiting substrate for the resident flora. Rapid digestion and absorption of starch, protein and fat from the small intestine effectively limits available substrate for the resident flora. Because enzyme supplementation reduces the microbial population in the small intestine (Choct *et al.*, 1995; Dunn, 1996), the entire gut ecosystem changes. These conditions in the gut alter the composition and activity of intestinal microflora (Vukic-Vranjes and Wenk, 1996). As dietary enzyme supplementation changes the gut microflora, there is a decrease in the adverse effects associated with microbial fermentation, such as 1) deconjugation of bile salts reducing fat digestion (Langhout, 1999); 2) competition between the host and the microflora for nutrients (Bedford, 1995; Choct *et al.*, 1996; Langhout *et al.*, 2000); and 3) atrophy of the intestinal villi and enlargement of digestive organs (Brenes *et al.*, 1993a,b; Viveiros *et al.*, 1994). Dietary enzyme supplementation has been shown to significantly reduce enterobacteria and gram-positive cocci in the small intestine of chicken, while counts of lactic acid bacteria increase in the same animal (Vahjen *et al.*, 1998).

Commercial NSP enzymes products are typically a blend of several different enzymes. For example, Allzyme Vegpro™ (Alltech Biotechnology, Inc.) is a solid-state fermentation product that contains a natural blend of enzymes, including protease, cellulase, pentosanase, alphagalactosidase, and amylase. Kumprencht *et al.* (1998) evaluated the performance of Ross broiler chicks fed diets containing Allzyme Vegpro™ at rates of 0, 325, 650, 1000, 1350 or 1650 g/ton of complete diet. After 42 days, birds supplemented with Allzyme Vegpro™ had increased body weight and improved feed conversion. It was determined that the optimum level of Allzyme Vegpro™ addition was 1000 g/tonne. These results were similar to findings reported by Prabhakar and Darur (1998) in India when feeding laying hens diets receiving 0 or 1 kg of Allzyme Vegpro™ per tonne of feed. Recently in Brazil, Teixeira *et al.* (2001) conducted a trial to evaluate the potential of using large amounts of soya with added Allzyme Vegpro™ in pre-starter pig diets. Allzyme Vegpro™ (4 to 6 kg/tonne) increased weight and feed consumption and improved the feed conversion ratio (FCR) of piglets at 28 days of age. Similarly, Fibrozyme, another enzyme preparation produced by Alltech Biotechnology Inc. has shown positive results in animal performance. Fibrozyme contains xylanase and cellulase, which has been shown to improve growth performance and carcass characteristics of pigs (Landblom *et al.*, 2005) and steers (Gomez *et al.*, 2003).

Taking in consideration that enzymes can modify the intestinal microflora to more favorable populations for the host growth, Parker *et al.* (2004) evaluated the utilization of a combination of amylase, protease, and xylanase designed for corn-soybean meal diets (Avizyme® 1502) as a feed additive to improve the responses of broilers vaccinated against coccidia in broiler starter diets with different levels of crude protein (CP). The normal variations in proportions of corn and soybean necessary to obtain three dietary protein levels were used to observe the efficacy of the enzyme under different substrates. There were 12 treatments in a 3 x 3 factorial, plus 3 negative

controls (No additives-No challenge) within each CP level were distributed. The CP levels (19, 21, 23%) and the anticoccidial control programs (Cocci-Vaccine=CV, Antibiotic + Ionophore, and Cocci-vaccine+Enzyme=CV+E) were evaluated as main effects. The chickens in the CV and CV+E groups were vaccinated against coccidia at 1 d of age with Advent<sup>®</sup>. All chickens, except those in negative control treatments were gavaged at 17 d with *E. acervulina*, *E. maxima*, and *E. tenella*. The enzyme product improved BW in vaccinated birds at 19 and 21% crude protein diets, but not at 23% CP. Lesion scores were affected ( $P<0.05$ ) by anticoccidial programs. Vaccinated chickens fed diets with enzyme had the lowest lesion scores in the caecum, while the antibiotic+ionophore group had the lowest lesion scores in midgut.

Coccidia challenge reduced the ileal digestibility of CP and amino acid (Oviedo et al., 2005f) by 7%. The enzyme addition did not improve significantly the ileal digestibility of coccidian vaccinated broilers. The modifications in microbial communities were measured by %G+C profiles and IgA concentrations were measured with ELISA (Oviedo et al., 2005a). Microbial responses were dependent on dietary CP level. The Coccidia challenge alone had very small effect on microbial numbers in the ileum, but suppressed IgA production. The addition of enzyme increased ileal microbial numbers at the lower CP level. In the caecum, the coccidia challenge reduced microbial numbers when compared with the non-challenged controls. CV+E resulted in microbial numbers and %G+C profiles similar to the control; especially at 19 and 21% CP.

These results indicated that the beneficial effects of this complex enzyme combination over BWG (5.6 and 17.0 %) and FCR (4.9 and 8.6%) 7 days after mixed coccidia challenge, in diets containing 18.5 and 20.1% of CP, respectively, might be due to changes in carbohydrate substrates available to microflora communities in the ceca. This has beneficial effects against *E. tenella*, and finally in live performance.

## Herbs, Spices and Essential Oils

Herbs, spices and essential oils (EO) have been used to make human foods more appetizing for centuries, and many of them are recognized for their health benefits. It is difficult to distinguish between them, because an EO is a mixture of fragrant, volatile compounds, named after the aromatic characteristics of plant materials from which they can be isolated (Oyen and Dung, 1999). The term 'essential' was adapted from the theory of 'quinta essentia' proposed by Paracelsus, who believed that this quintessence was the effective element in a medical preparation (Oyen and Dung, 1999). Because the term, 'essential oil' is a poorly defined concept from medieval pharmacy, the term 'volatile oil' has been proposed by Hay and Waterman, (1993) to be more appropriate. However, the name of 'essential oil' is still preferentially used. Essential oils are very complex mixtures of compounds and their chemical compositions and concentrations of individual compounds are variable. For example, the concentrations of two predominant components of thyme EO (i.e. thymol and carvacrol) have been reported to range from as low as 3% to as high as 60% of total EO (Lawrence and Reynolds, 1984). Cinnamaldehyde, a main component of cinnamon essential oil, amounts to approximately 60 to 75% of the total oil (Duke, 1986). Because of the large variation in composition, the biological effects (Schilcher, 1985; Janssen *et al.*, 1987; Deans and Waterman, 1993), if any, of EO may differ.

Essential oils have long been recognized for their anti-microbial activity (Lee *et al.*, 2004a), and they have gained much attention for their potential as alternatives to antibiotics. Lee and Ahn

(1998) found that cinnamaldehyde, derived from the cinnamon EO, strongly inhibits *Clostridium perfringens* and *Bacteroides fragilis* *in vitro* and moderately inhibits *Bifidobacterium longum* and *Lactobacillus acidophilus* isolated from human. Also, a wide range of *in-vitro* anti-microbial activities of essential oils derived from cinnamon, thyme and oregano have been published (Deans and Ritchie, 1987; Lee *et al.*, 2004a). The exact anti-microbial mechanism of EO is poorly understood, it may be associated with their lipophilic property and chemical structure (Lee *et al.*, 2004a).

Helander *et al.* (1998) investigated how two isomeric phenols, carvacrol and thymol, and the phenylpropanoid, cinnamaldehyde, exert their antibacterial effects on *E. coli* O157 and *S. typhimurium*. Both carvacrol and thymol disintegrated the membrane of bacteria, leading to the release of membrane-associated materials from the cells to the external medium. Conversely, cinnamaldehyde exhibited its antibacterial activity due to its lipophilicity of terpenoids and phenylpropanoids, which can penetrate the membrane and reach the inner part of the cell and impair bacterial enzyme systems. Therefore, these plant-based have antimicrobial effects similar to antibiotic compounds produced by fungi. As with antibiotics, continued use of these plant-based antimicrobials may result in the development of resistance in some pathogenic bacteria. However, more research is necessary to confirm this risk. In addition, to be as effective as growth promoters, these herbal antimicrobial compounds must be supplemented to the feed in a more concentrated form than found in their natural state, which may cause some economical disadvantage.

The EO supplementation has been tested in numerous studies with variable responses, depending upon the type of basal diet used, field or induced disease challenge, and other stress factors (Table 1). The most consistent positive response to EO blends has been observed under coccidia challenge. Oviedo *et al.* (2005d) evaluated the effects of two specific essential oil (EO) blends Crina<sup>®</sup> POULTRY (CP) and Crina<sup>®</sup> ALTERNATE (CA) on mixed *Eimeria* spp. infection in chickens vaccinated or not against coccidia with Advent<sup>®</sup>. The results 7 days after the mixed coccidia challenge with pathogenic viable oocysts showed that the non cocci vaccinated chickens fed the EO blend CA, and the cocci vaccinated chickens fed diets without feed additives had similar feed conversion ratios and body weight gains to the unmedicated-uninfected control broilers. The EO CP help to reduce coccidia lesion scores in duodenum and the EO CA reduced lesions in the caecum. The dietary supplementation of these EO blends did not improve any response in broilers vaccinated at first day of age.

The same treatments were evaluated (Oviedo *et al.*, 2005e) in a floor pen trial with used litter and previous *Eimeria* oocysts presence. Non-cocci-vaccinated chickens fed Crina<sup>®</sup> POULTRY had better feed conversion ratio (FCR) than the unmedicated control treatment in the starter period. The same EO improved FCR in coccidia vaccinated birds in the finisher period in comparison to the negative control group, but those responses were not significantly different from other treatments, or significant at 49 d of age. No significant differences were observed in coccidia lesion scores at 37 d. Diets supplemented with the growth promotant antibiotic (BMD) and the ionophore monensin supported consistently the best BWG and FCR in each period, and entire grow-out. No significant beneficial or deleterious effects on live performance were observed due to these specific EO blends in coccidia vaccinated broilers. It was concluded that these two specific EO blends differ in their efficacy to promote growth and the BMD+monensin combination was more effective on promoting better live performance.

**Table 1.** Literature review of the effects of specific essential oil blends on live performance, carcass traits and digesta parameters.

Essential oil composition	Diet, husbandry and challenge conditions	Effect of EO	Reference
Blend non specified	+ 20 mg/kg virginiamycin. Three experiment in cages	No effect on BW and FCR	Vogt, 1990, 1991
Crina <sup>®</sup> Poultry	Floor pens	No effect on BW and FCR	Ristic and Damme, 2001
Oregano (carvacrol – thymol)	Wheat-soybean meal basal diet – floor pens	No effect on BW and FCR	Botsoglou et al., 2002
Carvacrol and cinnamaldehyde combination		Negative effects as compared to the two fed individually	Lee et al., 2004c
EO blends		Improve antioxidant activity in carcasses	Dorman et al., 1995; Saricoban and Ozcan, 2004; Basmacioglu et al., 2004
Apacox <sup>®</sup>		No effect on BW and FCR, retarded lipid oxidation in meat	Botsoglou et al., 2004
Genex <sup>®</sup> (EO + organic acids) 500 – 1000 ppm		Growth promoting effect compared to flavomycin	Spais et al., 2002
Capsaicin, carvacrol, cinnamic aldehyde, 150 – 300 ppm	Wheat-barley diet	Improvement in BW (5.4, 8.1%) and feed conversion (3.1 and 7.1%). Reduced <i>E. coli</i> and <i>C. perfringens</i>	Jamroz et al., 2003
Crina <sup>®</sup> Poultry; or thymol and cinnamaldehyde	Maize-maize starch-soybean meal diet in wire floored cages	No effect of BW and FCR. Amilase activity improved in Crina <sup>®</sup>	Lee et al., 2003
Cinnamaldehyde	Diet with carboxymethyl cellulose, rye	Reverse negative effects of high viscosity diets	Lee et al., 2004 a, b
Blend from Turkey	Corn-soybean meal diets with wheat and sunflower meal in floor pens	Improved BW, FCR and carcass yield	Alçiçek et al., 2003
Crina <sup>®</sup> Poultry and combinations with antibiotics and lactic acid		Improve BW, FCR Combination with antibiotics or lactic acid did not show any effect	Suk et al., 2003
EO blend plus lactic acid		Improve BWG and increase digestive enzyme activities of the pancreas nad intestinal mucosa	Jang et al., 2004
Oregano (carvacrol and thymol)		Reduced feed intake and significantly improve feed efficiency	Halle et al., 2004
EO blend		Improve BWG, FCR and carcass yield	Alçiçek et al., 2004
RepaXol <sup>™</sup> (oregano, cinnamon, thyme, and capsicum) 100 ppm to 300 ppm.	Corn soybean meal diets in floor pens and cages from 1 to 42 d	No effect on BWG, FCR or FI. Improvement of final carcass yield and Feed efficiency at 14 d but not over the course of the experiment	Zhang et al., 2005

Crina® Poultry and Crina® Alternate	Corn soybean meal diets in floor from 1 to 49 d. Cocci-vaccinated birds	Improvement of FCR 13 d but not over the course of the experiment. No effect on cocci-vaccinated birds	Oviedo et al., 2005, e
Oregano (carvacrol and thymol)	Eimeria tenella infection - cages	Improve responses	Giannenas et al., 2003
Oregano (carvacrol and thymol)	Mixed Eimeria vaccination	Improvement in BWG and FI	Waldenstedt, 2003
Oregano (carvacrol, thymol)	Mixed Eimeria infection and induced necrotic enteritis	Improve BWG, lesion scores	Saini et al., 2003
Thymol, carvacrol, eugenol, curcumin, and piperin.	12 field trials to test Clostridium perfringens numbers	Reduced Clostridium perfringens colonization	Mitsh et al., 2004
Crina® Poultry and Crina® Alternate	Mixed Eimeria vaccination and infection	Crina Alternate but not Crina Poultry improved BWG, FCR and lesion scores post-infection. No effect on cocci-vaccinated birds	Oviedo et al., 2005, b, c, d,

Oviedo et al. (2005c, d) also measured the dynamics of intestinal microbial communities (MC) by DGGE and analyses of similarity coefficients (SC), in broilers vaccinated at first d of age with live oocysts of *Eimeria spp* and fed diets supplemented with the EO blends CP and CA. These broilers were challenged with pathogenic oocysts at 19 days of age. The dendrograms of amplicon patterns indicated MC differences due to intestinal location, feed additives, and cocci infection. The EO blends CP and CA did affect MC in all gut sections. The cocci-infection caused drastic MC population shifts in duodenal, ileal, and cecal sections (36.7, 55.4, and 36.2% SC, respectively). The CP supplemented birds had higher SC between pre- and post-challenge MC in duodenum and ileal (73.3, 81.8 %) than cocci-vaccinated chickens fed diets without feed additives (66.4, 66.5 %). However, cocci-vaccinated broilers had the smallest changes in cecal MC after challenge (79.5% SC). We concluded that cocci-vaccination causes small changes in intestinal MC, but challenge causes drastic shifts. The EO blend supplementation modulates MC in cocci-vaccinated broilers avoiding drastic shifts after a mixed challenge. The microbial ecology dynamics of the cecal compartment seems to be more related to the final broiler performance under conditions of stress.

## Acidifiers and organic acids

Acidifiers and organic acids have been used for decades in feed preservation, protecting feed from microbial and fungal destruction or to increase the preservation effect of fermented feeds (e.g. silages). Because organic acids have strong bacteriostatic effects, they have been used as salmonella-control agents in feed and water supplies for livestock and poultry. The most common organic acids in animal nutrition are citric acid, propionic acid, fumaric acid, lactic acid, formic acid and benzoic acid. Additionally, some other available acidifiers and organic acids have been shown to have some antimicrobial activity (Russell, 1992).

Experiments with pigs have shown that several organic acids, including citric acid, fumaric acid, formic acid, and propionic acid, have a positive influence on growth performance (Partanen and Mroz, 1999). It has been reported that the nutritive effect of organic acids is most pronounced in

weaning pigs (Gabert & Sauer, 1994; Roth & Kirchgessner, 1998), which often suffer from digestive disturbances resulting in diarrhea related to infections with *E. coli*. Problems at weaning may be triggered by an insufficient production of hydrochloric acid and digestive enzymes, and the feeding of a pre-starter diet with high protein content (Eidelsburger, 1997). Dietary acidification increases gastric proteolysis and protein and amino acid digestibility. The acid anion has been shown to complex with Ca, P, Mg and Zn, which improves the digestibility of these minerals. Furthermore, organic acids serve as substrates in the intermediary metabolism (Kirchgessner & Roth, 1988).

The use of organic acids has not gained as much attention in poultry production as it has in pig production, partly because limited positive responses in weight gain and feed conversion (Langhout, 2000). However, Vogt *et al.* (1982) reported a positive influence on either feed conversion ratio or growth performance by dietary supplementation of fumaric acid, propionic acid, sorbic acid and tartaric acid. Organic acids have mainly been used to sanitize the feed and reduce *Salmonella* colonization in poultry (Iba and Berchieri, 1995; Thompson and Hinton, 1997). An objective of dietary acidification is to inhibit of intestinal bacteria competing with the host for available nutrients, and reduce toxic bacterial metabolites (e.g. ammonia and amines).

The antibacterial activity of organic acids is related to the reduction of pH, as well as their ability to dissociate, which is determined by the pKa-value of the respective acid, and the pH of the surrounding milieu. The antibacterial activity increases with decreasing pH-value. Organic acids are lipid soluble in the undissociated form, and they easily enter the microbial cell by both passive and carrier-mediated transport mechanisms. Once in the cell, the organic acid releases the proton H<sup>+</sup> in the more alkaline environment, resulting in a decrease of intracellular pH. This influences microbial metabolism, inhibiting the action of important microbial enzymes and forces the bacterial cell to use energy to export the excess of protons H<sup>+</sup>, ultimately resulting death by starvation. In the same matter, the protons H<sup>+</sup> can denature bacterial acid sensitive proteins and DNA. Generally lactic acid bacteria are able to grow at relatively low pH, which means that they are more resistant to organic acids than other bacterial species, such as *E. coli* and *Salmonella*. Lactic acid bacteria, like other gram-positive bacteria, have a high intracellular potassium concentration, which counteracts acid anions (Russell & Diez-Gonzalez, 1998).

As with antibiotics, continued use of acidifiers and organic acids may result in the development of resistance in some pathogenic bacteria. Inducible resistance (adaptation or tolerance) to acidic environments is recognized as an important survival strategy for many prokaryotic and eukaryotic microorganisms. In addition, different microorganisms have developed different acid survival strategies. Inducible acid resistance has been observed in many gram-negative and gram-positive microorganisms. Kwon and Ricke (1998) suggested that SCFA in the gastrointestinal tract of a host animal or in food materials might contribute to the enhancement of the virulence of *S. typhimurium* by increasing acid resistance. Although bacteria under *in vitro* conditions are known to adapt to acids, it is not known whether this also occurs in GIT of animals fed organic acids.

## Probiotics

A probiotic is defined as “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance” (Fuller, 1989). *Lactobacillus* and *Bifidobacterium* species have been used most extensively in humans, whereas species of *Bacillus*, *Enterococcus*, and *Saccharomyces* yeast have been the most common organisms used in livestock. Live yeast is a probiotic, but yeast cell-wall is prebiotic (discussed later). Probiotics have a similar mode of

action as prebiotics, because both increase the colonization of symbiotic bacteria at the lower gut. Probiotic microorganisms inhibit growth of potentially pathogenic microorganisms by: 1) lowering the pH through production of lactate, lactic acid and SCFA; 2) competing for gut lining attachment and available nutrients; 3) producing bactericins; and 4) stimulating the gut associated immune system. Thus, probiotics have been shown to improve performance, decrease mortality, and improve FCR of poultry and swine.

Most commercial probiotic products are composed of pure cultures of one or more microorganisms. Competitive exclusion products originating from adult intestinal microbiota are usually inoculated to 1-day-old chicks in order to control of *Salmonella* contamination. Combinations of prebiotics and probiotics are known as synbiotics (Patterson and Burkholder, 2003). Availability of prebiotics specifically targeting specific bacterial strains would enable the development of a symbiotic product blend. A symbiotic blend is especially important for strains of probiotic microorganisms with poor survival properties, and to enhance of the effect of the probiotic as a treatment of enteric disease.

Probiotics have several disadvantages in comparison to other modulators of enteric microflora. Relatively few species of microorganisms can be considered for use in probiotics products due to their limited knowledge of culturability. Probiotics have a short shelf-life and most are labile to excessive heat and pressure during feed processing. Some probiotic microorganisms may be reduced or eliminated by the low pH in the gizzard, and thus have little effect in the lower intestinal tract where pathogens pose problems. If a probiotic is added to the drinking water, the chlorine sanitizer may adversely affect its survivability. Acidification would be a better sanitizer than chlorine when delivering a probiotic via the drinking water. Coating technology has helped with some of these concerns, but more research is needed.

## Prebiotics

Prebiotics are dietary components that are not indigestible to the host, but are readily fermented by many bacteria, predominant those that produce SCFA. Prebiotics have several advantages over probiotics, where culture viability needs to be maintained. Many important commensal bacteria that are present in a “healthy gut” cannot be cultured, so they cannot be used in commercial probiotic products. However, dietary supplementation of prebiotics has been shown to stimulate these unculturable bacteria in humans (Rastall *et al.*, 2005), and pigs (Konstantinov *et al.*, 2003). Moreover, prebiotics have the advantage of being more stable to the heat and pressure incurred during feed processing. Prebiotics also have an economical advantage because some of the best prebiotics are derived from inexpensive food processing by-products (Playne and Crittenden, 1996).

Prebiotics have great potential to modulate colonic microflora and discourage the colonization of enteric pathogens. Any food or feed ingredient that enters the large intestine is a potential prebiotic, but it must be fermented by microorganisms that benefit the host to be an effective prebiotic (Lan *et al.*, 2005). Most current attention and successes have been derived using non-digestible oligosaccharides, especially those that contain fructose, xylose, galactose, glucose and mannose (Gibson and Roberfroid, 1995; Gibson, 1998). It has been reported that oligosaccharides and polysaccharides are preferentially utilizable by *Bifidobacteria* (Yazawa *et al.*, 1978). A prebiotic substrate is selectively utilized by symbiotic bacteria of the gut but not potential pathogens, such as toxin-producing clostridia, proteolytic bacterioides and toxigenic *E. coli*. In this manner, a “healthier” microflora composition is obtained whereby the

*Bifidobacterium* and/or *Lactobacillus* become predominant in the intestine and exert possible health-promoting effects.

The dominant prebiotics are fructooligosaccharide products (FOS, oligofructose, and inulin). However, trans-galactooligosaccharides, glucooligosaccharides, glycooligosaccharides, lactulose, lactitol, maltooligosaccharides, xylo-oligosaccharides, stachyose, raffinose, and sucrose thermal oligosaccharides have also been investigated (Monsan and Paul, 1995; Orban *et al.*, 1997; Patterson *et al.*, 1997; Piva, 1998; Collins and Gibson, 1999). Although mannan-oligosaccharides (MOS) have been used in the same manner as the prebiotics listed above, they do not selectively enrich for beneficial populations. Instead, they act by binding and removing pathogens from the intestinal tract and stimulation of the immune system (Spring *et al.*, 2000).

In humans and animals, prebiotics has been widely studied on their ability to improve resistance to pathogens. A recent study in mice has shown that dietary supplementation of fructooligosaccharides and inulin was protective against enteric and systemic pathogens and tumor inducers (Buddington *et al.*, 2002). This includes the verocytotoxin strain of *Escherichia coli* O157:H7 and *Campylobacter*. Likewise, we have recently observed that a high concentration of plant cell wall polysaccharides in wheat-based turkey diets shifted the intestinal microflora of poults to a healthier state, and decreased *Salmonella* spp. population in the cecum (Santos *et al.*, 2005). The major effect of selective-fermentation prebiotics is that they increase lactic acid producing bacteria and short chain fatty acids (SCFA) in the ceca, which decrease the gastro-intestinal tract (GIT) pH. In effect, fermentative-prebiotics indirectly have the antimicrobial effect of an organic acid on susceptible gram-negative organisms as described above. Swanson (2002) observed that prebiotics affected the immune function of human and dogs by stimulating lactic acid bacteria. The rise in intestinal lactic acid bacteria stimulated phagocytic activity (cellular immune response) and/or IgA secretion (humoral immune response) that may affect the colonization of pathogens, such as *Salmonella* and rotavirus (Manning and Gibson, 2004).

### ***Mannan oligosaccharide (MOS)***

Unlike fermentative-prebiotics, MOS is not used as a substrate in microbial fermentation, but it still exerts significant growth-promoting effect by enhancing the animal's resistance to enteric pathogens. Bio-Mos<sup>®</sup> (Alltech<sup>®</sup>, Nicholasville, Kentucky, USA) is a commercial source of MOS that has been used in most of the published research literature. Based on the scientific literature, Bio-Mos<sup>®</sup> enhances an animal's resistance to enteric disease and promotes growth by the following means: 1) it inhibits colonization of enteric pathogens by blocking bacterial adhesion to gut lining; 2) it enhances immunity; 3) it modifies microflora fermentation to favor nutrient availability for the host; 4) it enhances the brush border mucin barrier; 5) it reduces enterocyte turnover rate; and 6) it enhances the integrity of the gut lining.

Mannan oligosaccharides, derived from mannans on yeast cell surfaces, act as high affinity ligands, offering a competitive binding site for a certain class of bacteria (Ofek *et al.*, 1977). Gram-negative pathogens with the mannose-specific Type-1 fimbriae attach to the MOS instead of attaching to intestinal epithelial cells and they move through the gut without colonization. Dietary MOS in the intestinal tract removes pathogenic bacteria that could attach to the lumen of the intestine (Newman, 1994). Mannose was shown by Oyofu *et al.* (1989a) to inhibit the *in vitro* attachment of *S. typhimurium* to intestinal cells of the day-old chicken. Then Oyofu *et al.* (1989b) provided evidence that dietary D-mannose was successful at inhibiting the intestinal colonization of *S. typhimurium* in broilers. The ability of MOS to interfere with the attachment of

pathogenic bacteria in the gut raises the possibility that it could also inhibit the binding between bacteria that is required for plasmid transfer via conjugation. This kind of inhibition of plasmid transfer in the digestive tract of mice colonized with human microflora has been described using lactose (Duval-Iflah, 2001). Lou (1995) demonstrated that dietary MOS supplementation decreased the proportion of specific groups of Gram-negative antibiotic-resistant fecal bacteria in pigs.

In an effort to confirm that MOS inhibits pathogens colonization, Spring *et al.* (2000) screened different bacterial strains for their ability to agglutinate mannanoligosaccharides in yeast cell preparations (*Saccharomyces cerevisiae*, NCYC 1026). Five of seven strains of *E. coli* and 7 of 10 strains of *Salmonella typhimurium* and *S. enteritidis* agglutinated MOS and *Sac. cerevisiae* cells. However, strains of *S. choleraeuis*, *S. pullorum*, and *Campylobacter* did not lead to agglutination. Although MOS does not bind clostridia, it does reduce clostridia numbers in some trials, possibly by enhancing the mucin barrier or stimulating gut-associated immunity.

MOS has been shown to have a positive influence on humoral immunity and immunoglobulin status. Good immune response is a nutritionally more efficient mean to resist disease than an active inflammatory response (Humphrey *et al.*, 2000). Savage *et al.* (1996) reported an increase in plasma IgG and bile IgA in poults fed diets supplemented with 0.11% MOS. An increase in antibody response to MOS is expected because of the ability of the immune system to react to foreign antigenic material of microbial origin. Portions of the cell wall structure of the yeast organism, *Saccharomyces* contained in MOS has been shown to elicit powerful antigenic properties (Ballou, 1970). However, MOS may also enhance humoral immunity against specific pathogens by preventing the colonization leading to disease, yet allowing them to be presented to immune cells as attenuated antigens. Indeed as MOS facilitates the secretion of IgA into the gut mucosa layer, pathogenic agents become more labile to the phagocytic action of gut-associated lymphocytes.

All animals reared under commercial field conditions are subjected to immunological stress depending on the pathogen load in their environment and the vaccination program. The release in cytokines associated with inflammation and the innate immune response results in fever (which reduces appetite), causes the mobilization of body reserves (glucose, aminoacids, and minerals) away from liver, muscle and bone, suppresses nutrient absorption in the gut, and increases body fluid losses as diuresis and diarrhea. The positive growth-performance effects observed among animals fed MOS may be partly due to its effect on acute immunological stress. Although MOS may enhance humoral immunity, there is some evidence that it may suppress the pro-inflammatory immune response that is detrimental to growth and production. To test this hypothesis, Ferket (2002) induced an acute immune stress in 14 day-old turkey poults by intraperitoneal injection of lipopolisaccharides (LPS) from *Salmonella typhimurium* strain SL 684. The poults were fed either 1 kg Bio-Mos<sup>®</sup>/tonne, 20g virginiamycin/tonne, or control diet from day of age. Cloacal temperatures were measured 8 hrs after the LPS injection, and then body, spleen, bursa of Fabricius, and intestinal tract weights were recorded 24 hours post-injection. In contrast to the control and the antibiotic-fed birds, the Bio-Mos<sup>®</sup>-fed birds showed no fever response 8 hrs post-injection, even though liver and intestine weights were increased. In other words, the MOS-fed birds retained normal body temperature after exposure to a pro-inflammatory antigen, while the controls and virginiamycin-fed birds expressed elevated body temperature. Under commercial conditions where birds are subjected to chronic immunological stress, MOS may help reduce the pro-inflammatory response and associated depression in feed intake and growth.

Even though the ceca are the primary site of gut microflora fermentation, microbial fermentation in the jejunum has a greater influence on digestion and nutrient absorption. Measurement of SCFA content and pH of the jejunum digesta is one way to evaluate the influence of feed additives on microbial fermentation. In a study with turkeys, Ferket (2002) observed that dietary supplementation of Bio-Mos<sup>®</sup> and antibiotics reduced total SCFA content of jejunum digesta by about 40%. Most of this effect was attributed to a reduction in propionic acid, which is the major fermentation product of microflora that uses starches and sugars as their primary substrate. Therefore, Bio-Mos<sup>®</sup> may improve dietary energy availability by reducing the microflora-host competition for available starches and sugars. Indeed, apparent metabolizable energy of the diet was increased by about 3% when Bio-Mos<sup>®</sup> or virginiamycin was supplemented to the diet. Another benefit to dietary inclusion of Bio-Mos<sup>®</sup> was a decrease in jejunum digesta pH and ammonia concentration in comparison to the antibiotic-fed birds. Lower gut pH suppresses the proliferation of putrefying bacteria that excrete ammonia as their fermentation byproduct, and ammonia has a detrimental effect on the integrity of gut tissues.

The beneficial effects of Bio-Mos<sup>®</sup> on the gut microflora, nutrient utilization, and growth performance may be associated with the brush border morphology and how it influences enteric disease resistance. To test this hypothesis, Ferket (2002) conducted an experiment to ascertain effects of Bio-Mos<sup>®</sup> and virginiamycin on jejunum villi morphology. Commercial Hybrid<sup>®</sup> poulters were fed a corn-soya control diet or diets supplemented with 1kg Bio-Mos<sup>®</sup>/tonne or 20g virginiamycin/tonne starting a 1 day of age. At 14 days of age, eight birds per treatment pen were sampled for morphometric measurements, including villus height, crypt depth, muscularis thickness, and goblet cell number. MOS had the greatest effect on villi morphology. Although MOS did not affect villus height, a decrease in crypt depth approached significance and villi height: crypt depth ratio was significantly greater than the control or VM treatment. Iji *et al.* (2001) also observed an increase in jejunal villi height: crypt depth ratio by MOS supplementation in broilers, but this was due to a significant increase in villi height rather than crypt depth. These researches also observed MOS to significantly increase protein/DNA of jejunal mucus, as well as increase in the brush border enzymes maltase, leucine aminopeptidase and alkaline phosphatase. Turkeys receiving MOS in our experiments also exhibited a thinner muscularis layer and increased the number of goblet cells per mm of villus height as compared to control birds.

The mucus gel layer coating the surface of the intestinal epithelium is the first barrier to enteric infection. Hence, the production of mucus, as indicated by the number of goblet cells, is an important feature in the protective scheme against pathogens. Feeding MOS resulted in an increased proliferation of goblet cells into the surface of the villus membrane. The innate immune system recognizes key molecular structures of invading bacteria, including lipopolysaccharides, peptidoglycans, and possibly the mannose structures in the cell walls of yeasts. Oligosaccharides containing mannose have been shown to affect the immune system by stimulating liver secretion of mannose-binding protein. This protein, in turn, can bind to bacteria and trigger the complement cascade of the host immune system (Newman, 1994). Intestinal microbes might influence goblet cell dynamics by releasing bioactive compounds or indirect activation of the immune system (Bienenstock and Befus, 1980).

## Conclusion

The intestinal tract is a major organ of the body, which contributes to critical functions of human and animal health, including the digestion and absorption of nutrients and the defense of the body's exposed surface against the external environment. The gastrointestinal tract is also host to billions of resident microflora that serve overall health that directly and favorably affect gut structure and function. Poultry are susceptible to the colonization of pathogens that are of concern to the human health. In response to consumer demands and government regulation, today's intensive animal agriculture industry is searching for alternatives to antibiotics. A combination of feed additives strategies and feed additives can be used to achieve good gut health and growth performance. Considering their flexibility of use and consistency in efficacies, mannan oligosaccharides supplied by a product such as Bio-Mos<sup>®</sup> with or without an enzyme preparation blend such as Allzyme Vegpro<sup>™</sup> are feasible alternative to promote gut health and growth performance.

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# 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*, *Bifidobacterium*, 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 multacidus*, 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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# **ASSESSING THE RESULTS OF THE EUROPEAN UNION BAN ON ANTIBIOTIC FEED ADDITIVES**

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## **INTRODUCTION**

The European Union (EU) banned the use of avoparcin, a widely used antibiotic feed additive in food-producing animals in 1997. The ban was carried out against the advice of the Scientific Committee on Animal Nutrition (21), a panel of experts composed of animal scientists from various EU countries. Two years later, the EU banned the use of bacitracin, spiramycin, tylosin and virginiamycin, once more the ban was carried out against the expert scientific advice of the SCAN (1, 22), citing fears of antibiotic resistance spread via the food chain and invoking the precautionary principle.

On January 1, 2006 the remaining antibiotic feed additives used in food-producing animals will be banned from use in the EU (13). Because several years have passed since the EU bans on antibiotic feed additives were implemented, and because some politicians in the USA are proposing a similar ban in this country (11, 24), it would seem appropriate to conduct an assessment of the ban results to determine if such bans have had a measurable effect on the problem of antibiotic resistance in human medicine. The purpose of this manuscript will be to evaluate and discuss published scientific information in regards to the EU bans on antibiotic feed additives and conclude if they have had a positive, a negative or no effect on the health of food-producing animals and on the problem of antibiotic resistance in human medicine.

## **BAN RESULTS ON ANTIBIOTIC RESISTANCE**

Most likely the oldest and most complete source of data regarding antibiotic use and antibiotic resistance monitoring in animals and people is the Danish database known as DANMAP, therefore, data from these reports will be used to illustrate the results and conclusions reached in this manuscript.

As anticipated, the antibiotic feed additive bans have resulted in substantially lower levels of antibiotic resistance for the corresponding antibiotic on indicator bacteria isolated from raw meat products. This should not surprise anyone, since it is a known fact that with a few exceptions, antibiotic use will create antibiotic resistance, whether in animals or people. What the DANMAP data shows, however, is that the improvements seen on indicator bacteria isolated from raw meats have not translated into lower levels of antibiotic resistance in human patients (10, 5, 9, 20). There is an abundant body of published scientific information that serves to explain this lack of correlation.

The first antibiotic feed additive used in food-producing animals banned in 1997 by the EU was avoparcin. This antibiotic was banned from use in food-producing animals because it belongs to the glycopeptide class, a critically important antibiotic used in human medicine, vancomycin, also belongs to this class and studies have shown that glycopeptide-resistant enterococci will develop in animals fed avoparcin (8), also resistant enterococci have been isolated from raw meat of animals fed avoparcin creating a concern for passage of resistant enterococci to people via the food chain (6). This is what led to the ban of avoparcin as an antibiotic feed additive in the EU.

However, when one examines the incidence of vancomycin-resistant enterococci (VRE, bacteria commonly involved in fatal infections in human hospitals), a different picture emerges. That is because VRE infections are far more prevalent in USA hospitals than in EU hospitals (1, 19), and since avoparcin has never been used as an antibiotic feed additive in food-producing animals in the USA, it must be concluded that 100% of the VRE problem has been created by vancomycin use in humans. Obviously, a ban on antibiotic feed additives in the USA would do nothing to improve the critical VRE problem in USA hospitals. Although studies in Europe have shown that VRE can be isolated from healthy human and animal feces, the relatively low prevalence of VRE in hospitalized EU patients suggests that without substantial use of vancomycin in human medicine, the VRE problem would be very limited (19,20).

Another antibiotic feed additive used in food-producing animals banned by the EU in 1999 is virginiamycin. This antibiotic belongs to the streptogramin class and as in the case of avoparcin, concerns over cross-resistance with a new human antibiotic in the same class, Synercid, developed for treatment of vancomycin-resistant *E. faecium* (VREF) infections prompted EU regulators to call for its ban as an antibiotic feed additive in food-producing animals.

However, a very extensive sensitivity survey conducted in American and Canadian medical clinics before Synercid use began in North America found that out of more than 1,000 clinical isolates of *E. faecium* tested, 99.8% were sensitive to the new human antibiotic (14). Therefore, this study showed that after nearly 3 decades of continuous use of virginiamycin in food-producing animals in the USA and Canada, there was virtually no evidence of streptogramin-resistant *E. faecium* (SREF) in the human population. These results are not surprising since meat is cooked prior to its consumption, and the high temperatures achieved during cooking kill any bacteria that might have contaminated it, and dead bacteria cannot transmit antibiotic resistance. The importance of proper food hygiene and cooking has been pointed out by others as the most effective way of preventing not only transmission of antibiotic-resistance bacteria but also of preventing food poisoning in people (20).

Another study published in 2001 in The New England Journal of Medicine (18) which was specifically designed to prove the transfer of SREF from foods of animal origin to people, failed to do so. Between July 1998 and June 1999, the authors cultured 407 raw chickens obtained from 26 grocery stores in 4 states, and isolated SREF from 58.2% of them. Resistance was defined as a minimum inhibitory concentration (MIC) of at least 4 ppm. The authors attributed the high level of resistance to the use of virginiamycin. During the same period the authors also cultured 334 stool samples from outpatients at

various medical clinics in the same 4 states. In contrast to the significant level of resistance found in the raw chickens, only 2 stool samples, or 0.6% of the total yielded SREF. It is worth noting that both samples had an MIC of 4 ppm reported by the authors as a “low level” resistance.

In spite of these results, the authors concluded that although “the low prevalence and low level of resistance in human stool specimens suggest that the use of virginiamycin in animals has not yet had a substantial influence”, “foodborne dissemination of resistance may increase”, they concluded by saying that the Food and Drug Administration (FDA) was in the process of conducting a risk assessment for virginiamycin and that if such assessment demonstrated a role for food borne transmission in the emergence of SREF in humans, restrictions on the continued use of virginiamycin in food animals should be considered”.

Since that manuscript was published, two risk analysis have been conducted. A quantitative risk analysis showed that the risk of the continued use of virginiamycin as an antibiotic feed additive in food-producing animals, assuming that transmission of resistance from animal foods to people occurs (an unproven assumption in this case), the risk would be less than one statistical life saved for the entire USA population over a 15 year period and rapidly decreasing by the increased use of newer antibiotics as alternatives to Synercid (7). FDA also completed its own risk assessment and also concluded that the risk from the continued use of virginiamycin in food-producing animals is very small. The FDA risk assessment concludes that with a food pathway attribution assumption of 10% the average risk to a random hospitalized member of the US population, the most relevant “at risk” population, of having SREF attributable to animal uses of virginiamycin and that may result in impaired Synercid therapy, ranges from 6 chances in 100 million to 1.2 chances in 1 million in one year, and that with a food pathway attribution assumption of 100% the chances would increase 10-fold. To present a comparative perspective on risk the following example is provided from an article on risk assessment of fluoroquinolone use in beef cattle (2), a study had estimated approximately a 1-in-250 million chance that a person could die from a case of *Campylobacter jejuni* infection that is resistant to fluoroquinolone antibiotics, which the person might have caught by eating contaminated ground beef. In comparison to this risk, in any given year a person is 567 times more likely to be killed in a plane crash and 14,284 times more likely to be killed in a car crash.

It is because of all of this and the thorough examination of many other published research reports, that a panel of experts concluded that “there is little or no evidence that resistant enterococci from animals are a risk to human health, and that a ban of growth promoting antibiotics was not justified on this basis, and will have no impact on the prevalence of VRE in human infections” (20).

## **BAN RESULTS ON ANIMAL HEALTH AND PRODUCTIVITY**

A manuscript by researchers from the National Veterinary Institute of Oslo, Norway (16) reported in 2001 severely impaired production performance in broiler flocks with high incidence of *Clostridium perfringens*-associated hepatitis (CPAH). The authors analyzed production performance data collected from a large processing plant in Norway, with the objective of comparing production performance data from broiler flocks with high levels

of CPAH to flocks with low levels of CPAH. The study was conducted for the first 2.5 years following the ban of avoparcin, the first antibiotic feed additive to be banned by the EU. This study showed that flocks with high levels of CPAH had 25 to 43% lower profitability than those with low levels. The authors cited impaired feed conversion and reduced weight at slaughter as the major causes for the losses. Researchers from the same Institute had reported earlier that the main effects of experimentally-induced subclinical necrotic enteritis were increased feed conversion and retarded growth rate (15). So it has become increasingly clear following the EU bans that the antibiotic feed additives, like avoparcin and virginiamycin, were preventing clinical and subclinical necrotic enteritis in poultry, even when used at inclusion rates labeled for “growth promotion”, this is in agreement with the observations made by others (20).

In another manuscript (5), the authors examined data 3 years after the bans were implemented and concluded that the only measurable benefit in humans was a reduction in acquired resistance in enterococci isolated from human fecal carriers, however, the authors stated that despite the growth promoter ban and the reduction of carriage of resistant enterococci in animals and humans, there had been no reduction in the prevalence of resistant enterococcal infection in humans. On the other hand, the authors also stated that the antibiotic feed additives had an important prophylactic activity previously unrecognized and that their withdrawal was now associated with a deterioration in animal health, evidenced by an increased incidence of diarrhea, weight loss and mortality in post-weaning pigs, and necrotic enteritis in broiler chickens. The authors closed by saying that “the theoretical and political benefit of the widespread ban of growth promoters needs to be more carefully weighed against the increasingly apparent adverse consequences”.

## **BAN RESULTS ON HUMAN HEALTH**

An unintended consequence of the EU ban on the prophylactic use of antibiotic feed additives in food-producing animals may have an even greater adverse effect on public health. In a manuscript published in December, 2004 (13) the authors indicate that following the EU bans, the incidence of *C. perfringens*-associated disease in poultry and its detection in poultry meat has increased substantially and it is emerging as a real threat to public health. According to the authors, toxins formed by *C. perfringens* type A and type C present in poultry meat can cause food poisoning and necrotic enteritis in people, respectively. Since *C. perfringens* food poisoning is not a reportable disease, its incidence is in all probability greatly underestimated. Nevertheless, *C. perfringens* was recognized in Norway as the most common cause of food poisoning during the decade of the 1990s (3). According to F.V. Immersel, et.al., 2004, with the ban of the remaining 3 antibiotic feed additives (avilamycin, monensin and salinomycin) with activity against *C. perfringens*, the public threat of *C. perfringens*-induced food poisoning is expected to increase even more. Time will tell the magnitude of the consequences of the bans on antibiotic feed additives as related to food poisoning in humans.

According to the latest available report by DANMAP, “the use of antibiotics in humans and animals and the occurrence of resistant bacteria continued to increase through 2004”. In the mean time, antibiotic use for therapeutic purposes in food-producing animals has

increased every year since the first bans, from 48,000 kilograms the year after the bans to 112,500 kilograms in 2004.

An interesting theory has recently been proposed on how antibiotic use in food-producing animals may actually reduce consumer risk (12), a professor of veterinary medicine provided various ways by which antibiotic feed additive use in food-producing animals may actually lower the risk of food poisoning in people. This seems to be in agreement with a recently published manuscript that indicated for example, that the use of virginiamycin in turkey feeds significantly reduced the incidence of *salmonella spp.* (7), since virginiamycin has no direct activity on *salmonella spp.*, we must assume that the changes produced in the intestinal microflora were less favorable to its growth. Likewise, the use of antibiotics, whether added to the feed to prevent disease or in the drinking water to treat diseases like airsacculitis of poultry, may also aid in reducing the risk of food poisoning to consumers. In a series of studies conducted to determine the effect of airsacculitis (an infection of the air sacs) of broiler chickens on the overall quality of the carcass (23), the researcher found that airsacculitis-positive flocks had lower body weights, more fecal contamination, more processing errors and higher levels of *Campylobacter spp.* The author concluded that broiler chicken companies should emphasize control of airsacculitis in the flocks as a means of preventing subsequent food borne bacterial infection.

Finally, it has recently been reported that concentrations of various antibiotic feed additives and ionophore anticoccidials similar to those normally used in poultry rations had an inhibitory effect on the transfer of a multiresistance-conferring plasmid in *E. coli* in an *in-vitro* test system (17). The authors concluded that based on the results of these tests, feed additive antibiotics and ionophore anticoccidials may actually inhibit resistance transfer mechanisms within poultry and livestock.

## CONCLUSIONS

There is little to no evidence to support the claim that the use of antibiotic feed additives in animals feeds has contributed to the problem of antibiotic resistance in human medicine. This conclusion is further supported by the fact that of the 20 most serious bacterial infections exhibiting problems with antibiotic resistance in human medicine, 12 are in no possible way related to antibiotic use in food-producing animals as these bacteria cannot be acquired via the food chain. Of the remaining 8, assuming that transfer of bacterial resistance from animals to people occurs (an unproven assumption in most cases), the calculated percent contribution to antibiotic resistance in all cases is 1% or less, in most cases less than 0.5% (4).

The EU banned the use of various antibiotic feed additives at levels labeled for growth promotion. Almost immediately a surge of enteric disease problems in food-producing animals followed. The surge in enteric diseases of food-producing animals was followed by a surge in antibiotic use in food-producing animals for therapeutic purposes. The antibiotics used to treat food-producing animals belong to the various classes of antibiotics most frequently used in human medicine, this might have actually had a more adverse effect on the creation of antibiotic resistance in people than the use of the antibiotic feed additives. The surge in use of antibiotics for therapeutic purposes in food-producing animals has clearly proven that the prior use of antibiotic feed additives had a

health promotional and disease prevention effect in food-producing animals even when used at concentrations labeled for “growth promotion”.

Although the antibiotic feed additive bans implemented by the EU achieved the objective of reducing the incidence of resistance on indicator bacteria in raw food products of animal origin, this has not resulted in any measurable improvement on the problem related to antibiotic resistance in human patients or human hospitals. This may be explained by the fact that monitoring of antibiotic resistance in raw meat products is not representative of the bacteria that may actually reach the consumer. Proper cooking of foods of animal origin destroys any bacteria that might have contaminated them, and dead bacteria cannot transmit antibiotic resistance to people.

While the incidence of food borne diseases in the USA population has continued to decline, in the EU it has continued to increase, at least for certain bacteria like salmonella, campylobacter and *C. perfringens*. Therefore, it is becoming increasingly apparent that the bans on antibiotic feed additives have not resulted in a safer food supply.

The USA should learn from the EU experience and proceed with caution and only make decisions supported by science and quantitative risk analysis rather than implementing bans that may actually have effects opposite to their intended ones.

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# IMPACT OF A BAN ON ANIMAL BY-PRODUCTS AND ANTIBIOTIC GROWTH PROMOTERS –EUROPEAN EXPERIENCE

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Over the last ten years the UK poultry feed industry has been subjected to a series of shifts in legislation and consumer demands that has completely changed the nature of the diets being used in the production of broilers and turkeys. Feed formulas that would have been recognisable to US producers containing animal proteins and fats, ten years ago, have now turned into ‘Veggie’ diets, using basically cereal, soyameal and vegetable fats. At the same time European politicians and legislators have gradually reduced the products available for use as antibiotic growth promoters to the point that from the end of 2005 there will be none of these additives left. Other legislation has also reduced the range of anti-coccidial products, treatment antibiotics, removed all anti-histomonas drugs and made it harder for new products to become approved.

These changes have come about following a series of scares in the food industry that have caused a shift in the mentality of the consumer. The consumer, – the housewife, has reacted to these food scares by making changes in their buying patterns.

The supermarkets who have huge purchasing power in Europe over the poultry producers have reacted to become the housewife’s champion, pushing through their own demands, over and above legislation, that the food going onto their shelves is ‘minimum risk’. The UK poultry producers, being weak sellers, have had no choice but to adopt all these measures, absorb any extra costs and find the best way to survive. Failure to comply has resulted in loosing market share to other European countries, or Thailand and Brazil.

## ***Food Scares – history.***

The first major foods scare hitting the poultry industry goes back to 1988 when a UK government minister made the statement: “...**sadly a high proportion of the laying flock is contaminated with salmonella...**” At this time *S. enteritidis* was rampant in the UK, especially in commercial layers. This statement, while true, was badly presented. The public reaction was a rapid reduction in egg consumption – 50% in the first week, and close to 30% as an ongoing loss in market share. The affect on the producers was devastating. The legislators then piled on the misery by increasing the statutory testing regime on flocks, followed by slaughter of positive birds.

A few years later in 1996 BSE (Bovine Spongiform Encephalopathy) hit the headlines. What had been an increasing problem that had not been controlled by normal measures suddenly became ‘Hot News’. The media took the story and turned it into a political issue. Initially politicians and their scientists claimed that the public were not at risk, but soon evidence of transmission emerged and vCJD (variant Creutz-Feldt Jacob Disease) identified in humans. Consumption of beef plummeted, legislators rushed through changes in identification of affected cattle and handling in the abattoirs. Once a potential culprit was identified – the feed, a ban on the use of all mammalian proteins in any animal feed was introduced. There has never been evidence of transfer to poultry, but they have been subjected to the same ban.

At this stage, the supermarkets began to realise a role in ‘protection’ of their customers. Science was now a dirty word and scientists not to be believed. Although Tallow had been shown to not carry any risk, they demanded that poultry producers should stop using

it. This was quickly followed by a thought process that feeding 'like to like' was not good news, so the next demand was for a halt in the use of poultry meal and poultry fats in any poultry diets.

The safety of food had become big news and the media pursued stories to sell newspapers. Over the next few years a number of food-related issues continued to hit the headlines and dent the consumer's perception of the agricultural industry. Examples of these from across Europe have been:

- E coli in beef - UK
- Dioxins in fats – Belgium
- Sewage in feed – France
- Hormone in molasses – Holland
- Herbicides in wheat – Germany
- Chloramphenicol in fishmeal – Germany
- Cadmium in minerals – Holland (ex USA)
- Veterinary residues in imported meat – China, Brazil, Thailand
- Foot and Mouth – UK
- Dioxins in Salmon – UK
- Residues in meat of banned additives – UK
- GM Crops – imported Maize and Soya products from USA.
- Avian Influenza – is currently scaring people away from poultry consumption.

The effect of these issues has been for further legislation on the feed industry. Fishmeal was banned from ruminant diets in 2000, due to suspected cross-contamination with mammalian proteins. Feed mills manufacturing ruminant and poultry diets had to remove fishmeal from all diets, until cross over could be ruled out. Some countries extended this to a total ban and some supermarkets or fast food chains requested the removal of fishmeal on environmental concerns of loss of resources. Recycled cooking oils can no longer be used in animal feeds. Clay binders have been banned.

At the same time as these pressures were building on the ingredients used in animal feeds, another pressure group was building based on the concerns of cross resistance developing in bacteria. Strains of organisms resistant to antibiotics, were beginning to be detected in human medicine. These 'super-bugs' were beginning to cause concerns in hospitals where doctors were not able to control some diseases in their patients.

Claims were made that the wide spread use of antibiotics in agricultural was out of control and that abuse of these drugs was allowing bacteria in animals to develop resistance and that these bugs were somehow transferring this resistance to bacteria in the same family, which were then affecting humans.

The use of specific antibiotics to improve the performance of animals when fed continuously had been going on since the 1940's. The risk of cross-resistance had been identified in the 1960's and at that time the range of products that could be used was restricted to a group of drugs having no cross over with humans. Sweden was the first country in Europe to completely ban the use of antibiotic growth promoters in 1985. This ban was actually requested by the organisation of Swedish farmers, and was subsequently enforced by the Swedish government. When Sweden joined the EU in 1994 it was granted a derogation, allowing the ban to continue in Sweden whilst other EU countries continued the use of antibiotic growth promoters. Sweden lobbied for an EU wide ban through and during the late 1990s. Following the other feed scares, the pressure built to such an extent that the EU began a programme of re-evaluation of all feed additives and a

campaign of removal of 'unsafe' products developed. Many times decisions were made without or with very limited scientific input, the actual vote being won on political rather than scientific grounds. Some companies have refused to reinvest in the registration process, finding that the hurdles set by the EU required large expenditure in re-proving the efficacy and safety of products. Even after that outlay there are no guarantees of approval and no assurances that the approval will last long enough to recover the investment before a new set of regulations might sweep them away.

### Products lost to UK industry

Active Ingredient	Activity	Date of Ban
Furazolidone	General antibiotic	1990?
Avoparcin	AGP	1996
Spiramycin	AGP	1999
Tylosin	AGP	1999
Zinc Bacitracin	AGP	1999
Virginiamycin	AGP	1999
Dimetridazole	Anti Histomonas	2000
Avilamycin	AGP	1999/2001/2004 Industry Ba
Bambermycin	AGP	1999/2001/2004 Industry Ba
Nifursol	Anti Histomonas	2003
Avilamycin	AGP	2006 EU Ban
Bambermycin	AGP	2006 EU Ban

### Coccidiostats that have disappeared from the market since 2002

Active ingredient	Brand name
Amprolium	Amprol
Amprolium/Ethopabate	Amprol Plus
Clopidol	Coyden
Clopidol/Methylbenzoquate	Lerbek
Nicarbazin	Various
Salinomycin	Generic versions
Monensin	Generic versions

### Growing Poultry without Animal By-products

In the space of 2 years the UK poultry industry had to learn to produce diets without any animal by-products. The ingredients which may have been used prior to this time where:

- Meat and Bone Meal
- Blood Meal
- Animal Tallow
- Poultry Meal
- Feather Meal
- Poultry Fat
- Fish/meat protein blends
- Fish Meal – mixed mills/some customers

What feed ingredient is still allowed to be used?

- Soyabean meal – Hipro 49%
- Full Fat soya – toasted, extruded or expanded
- Rapeseed extracted meal (Canola)
- Whole Rapeseed
- Sunflower meal – 30% or 38%
- Pulses – peas and beans
- Pulse/rapeseed blends (cooked)
- Potato protein meal
- Corn Gluten 60%
- DDS
- Biscuit / cooky meal
- Rice Bran
- Vegetable Fats
- Vegetable soap-stocks

What have been the consequences of removing these animal by-products and having to rely on vegetable proteins? Other than the protein and essential amino acid content, animal proteins are good sources of:

- Calcium
- Phosphorus – none phytate
- Trace Minerals –organic form
- Vitamin B12
- Choline
- Glycine
- Saturated fats
- Long chain fatty acids -fish
- Carnitine

However, because they are by-products they, they also have negative attributes, such as variable analysis; availability of some nutrients can be variable, depending on processing; and high microbiological risk.

Removal of these animal proteins happened in stages. Meat and Bone meals was removed from feeds very quickly after the BSE situation blew up in 1996. This initially required rebalancing of the formulas to be able to meet the diet specifications. But, in integrated companies with poultry meal and fishmeal still available, this presented very few difficulties as long as all the nutrients were rebalanced. Following this first change, performance of the birds improved, we saw an increase in growth and less variation in results between farms. This was attributed to the variability and variation in the quality of the meat and bone meals.

Companies that were not using poultry meal at this time experienced a few more problems in balancing diets, but they tended to rely on increased levels of fishmeal and vegetable protein, such as Full-Fat Soya and Canola meal. In 1997, when the poultry meal, animal tallow and poultry fat was forced out by consumer demand, the diets became quite different. Fishmeal remained as the only ‘Animal’ protein source, but due to its cost was only used routinely in starter and grower diets. From 2000, Fishmeal was banned in some countries and in mixed feedmills. Examples of how broiler starter and finisher diets evolved are shown in Tables 1 and 2, respectively. Examples of how turkey starter diets evolved are shown in Tables 3 and 4. These show the increased reliance on

mineral phosphates and synthetic amino acids and the use of vegetable oils to replace the animal fats. Following the removal of fishmeal, there has been a wider use of vegetable protein sources. Full-Fat Soya is used as a source of highly digestible fat, to replace some of the poultry oil and tallow. Another product that is now used widely in poultry diets is a combination (50:50 mix) of Canola seed and pulses, such as Field beans or peas. This is usually processed through an expander or extruder to make the oil fully available, and to destroy some of the anti-nutritive factors in the vegetable products.

In general terms growing poultry on ‘veggie’ diets has been achievable. There has been a need to balance out micro nutrients and this was a learning curve over the first few months. The following issues have arisen during this period:

1. Cannibalism increased, not as an increase in aggression, but as consumption of any mortality left for more than a few hours. This has been reduced by adjustment of phosphate levels and an increase in trace minerals. Moving to a digestible phosphorus system has resolved some of these issues. By increasing the reliance on vegetable proteins the amount of phytate phosphorus in the diet has increased. Also the use of increased levels of mineral phosphate has highlighted the importance of assigning digestibility values to different types of phosphate. Some of the cases of cannibalism have responded to the addition of chelated trace minerals, indicating that the animal proteins may have been providing these minerals in an organic form.

### Phosphorus digestibility in feed ingredients

Wheat	27%
Maize	17%
Barley	30%
Oats	27%
Hipro Soya	22%
Sunflower meal	19%
Canola Meal	28%
Fishmeal	81%
Meat & bone meals	95%
Poultry meal	90%
Midds	27%
Distillers Grains	25%
Rock Phosphate	72%
Mono calcium phosphate	84%
Dicalcium Phosphate	76%

2. Litter was initially harder to manage, with more cases of enteritis. During the changes in ingredient usage there were also changes in the use of AGP’s going on so the situation was quite complicated. However, some of these litter issues related to the ‘veggie’ diets was associated with electrolyte levels and the types of fat being used. By moving away from animal proteins, the balance of Sodium, Potassium and Chloride in the diets altered. Vegetable proteins, especially Soya are high in Potassium, and low in Sodium and Chloride. Simple adjustment of added salt or sodium bicarbonate back to the same levels did not appear to resolve the problems. Water intake on the vegetable diets does tend to be higher than on

diets using animal proteins. There is now a tendency to use lower sodium and chloride levels in the diets.

### **Electrolyte Levels in Ingredients**

	Sodium	Potassium	Chloride
Meat+Bone Meal	0.6	0.55	0.65
Poultry Meal	0.2	0.3	0.25
Fish Meal	1.3	0.7	2.0
Soya Hipro	0.04	2.1	0.05
Canola meal	0.01	1.3	0.07
Sunflower Meal	0.01	1.5	0.15

### **Electrolyte Balance in Turkey Starter diets**

Turkey Starter	Sodium	Potassium	Chloride	MEQ
Animal	0.15	0.95	0.23	258
Vegetable	0.14	1.15	0.18	297

In the move away from animal fats, blends containing high levels of vegetable oil soapstocks have been used, to try and provide a cost effective replacement. These can be quite variable in the levels of Free Fatty Acid (FFA) and Unsaponifiable materials.

Some samples of Palm Fatty Acid have been seen with FFA levels close to 90%. When these are included in blends the FFA level in some blends has been over 40%.

Young birds cannot handle these types of materials and the fat passes through the gut undigested and can also lock up some of the minerals as soaps. To maintain performance, it has been found necessary to use pure vegetable oils in diets for young birds and then to gradually move to the vegetable blends as the birds get older. The type of blends now in use will have a FFA content of no more than 25%.

### ***Growing Poultry without Antibiotic Growth Promoters (AGP)***

In the late 1990's, it was becoming clear that the pressure to remove AGPs was gradually increasing. The Scandinavian countries had begun to make the transition to removing these products because of the concerns about increased resistance of bacteria to certain antibiotics. When some of these countries joined the EU in 1994 they began to influence the thought processes of the law makers of the EU in Brussels. The poultry industry, realising what was coming, began to evaluate the effects of removing these additives. In small scale trials the actual changes in performance did not appear to be too detrimental. When one UK company announced that it had run its own trials and would remove all AGPs at the end of 1999 as a marketing strategy, the supermarkets immediately jumped on the band-wagon and demanded that all their chicken product suppliers should follow suit.

So we all plunged in, believing that our trial work showed the results were not going to be too bad. Minor adjustments were made to the diets, but there was no major shift in diet composition and probably no changes to management practices. Most companies would have been on mainly vegetable protein and vegetable oil diets, using some fishmeal in starter and grower diets, but no other animal products. For the first 6 months, performance was generally quite good and then as we got into the winter months the incidence of Necrotic Enteritis (NE) and wet litter gradually began to increase and the

number of rejects at processing began to rise. The use of antibiotics to treat the problems began to increase. Penicillin and similar drugs were being used to treat the NE and broad spectrum products were used against the enteritis problems. Growth of the birds tended to hold up quite well, but feed conversions (FCR) deteriorated significantly, by 10 to 15 points on some of the worse affected farms. Most producers struggled through that first winter supporting performance with antibiotics, expecting that as the warmer weather returned things would get better. They did but only marginally, the incidence of NE decreased, but the problems of the enteritis and wet litter continued.

A new phrase developed to describe the 'condition' – **Dysbacteriosis**, which became the description for what was being seen on the farm. It loosely describes the state where by there is a massive proliferation of bacteria in the gut of the bird. The response is for the bird to try to flush out the gut, resulting in high levels of water consumption. The droppings when seen on the litter tend to be very wet and visually appear as a normal type dropping, but very wet, surrounded by a large ring of water.

Gradually it dawned upon the industry that this was a problem that was not going to be resolved within existing systems. The removal of the AGPs had shifted the landscape: these products had maintained a level of bacterial stability that was not really evident until they had been removed. The poultry industry responded to the challenge and began to evaluate how to produce poultry without these additives.

After 5 years, I would say that the production and performance of poultry in the UK has never been better.

The approaches that have been taken and appear to be beneficial are:

1. Reduce the bacterial load going into the bird
  - a) Feed is now processes to achieve low levels of enterobacteriaceae. Targets are  $<10\text{cfu/g}$  after pelleting and  $<10^3\text{ cfu/g}$  on-farm
  - b) Maintain the litter in a dry and friable state – the depth of material is generally greater, more gas will be used to drive ventilation to keep the litter dry. It is important not to let bacterial levels build in the litter.
  - c) Match stocking densities to the capabilities of the ventilation system so that the litter is not overloaded.
  - d) Monitor water quality and ensure bacterial levels are kept low, through sanitisation.
2. Promote the development of gut health
  - a) Use of a probiotic to seed the gut with friendly bacteria is used in some companies.
  - b) Stimulate the physical activity of the intestines
    - Coarse grist of feed particles
    - Adding whole wheat as a gizzard stimulant
    - Meal feeding rather than free access
  - c) Enforced rest periods – minimum 4 hours dark
  - d) Increased use of feed ingredients with soluble fibre and reduction of insoluble fibre fractions.
3. Improved digestibility of feeds to reduce the supply of nutrients to lower intestine bacterial populations.
  - a) Use highly digestible fats especially in early diets

- b) Incorporation of exogenous enzymes - xylanase and phytase
  - c) Selecting of ingredients with a higher and faster digestibility of starch.
  - d) Minimisation of wheat in diets by use of alternative cereals – maize, oats, barley.
  - e) Reduce non-protein nitrogen levels by formulating based on digestible amino acids and through the use of higher levels of synthetic amino acids.
  - f) Adjust electrolyte levels to minimise stimulation of water consumption
4. Strategic use of coccidiostats and antibiotics.
- a) Selection of anticoccidial products that have the highest level of activity against clostridia, used in the right sequence at the vulnerable stages.
  - b) Reacting quickly to control enteritis by pre-empting problems: monitor water consumption, this will often increase at the start of a problem. Antibiotics are then given in a short sharp dose to clear the problem up.
  - c) Good husbandry, observe bird behaviour, feed selection/flicking is seen at the start of a problem. Treat the birds when this is seen.
5. Maintain tight biosecurity on farms.
- a) Sites should be single age all-in all-out.
  - b) Down time between flocks needs to be adequate, minimum 5 days.
  - c) Full cleanout, removal of litter, washing and disinfection.
  - d) If taking an undercrop out the hygiene of the catching gang needs to be controlled.
  - e) Movement of personnel from farm to farm increases risk

### ***AGP Replacement***

There are many products that have come into the market since the removal of the AGPs. There are many types and claims for these products. Some alternatives that are promising as partial replacements of AGPs include; probiotics, prebiotics, organic acids, enzymes, herbs, phenolic aromatic components and many others. However, AGPs appear to have a unique mode of action, and it is probably not possible to replace this with products that by their nature have a different mode of action. The bacterial populations within the gut appear to have been controlled very effectively by the AGPs. Alternative products do not seem to have as consistent effect in controlling the bacterial populations and this probably explains some of the lack of consistency in responses when these alternatives are evaluated under field conditions.

### ***Conclusions.***

Due to legislative and consumer demands the nature of the diets being fed to commercial poultry in Europe has changed significantly over the last 10 years.

Animal proteins and fats have been removed from the diets. The use of AGPs will cease completely from 2006, although the majority of broilers and turkeys have been produced without these additives for several years. Through a gradual learning process, diets have been manipulated to minimise the initial problems of increased enteritis. By promoting gut health and adjusting the balance and use of ingredients in diets birds are now being successfully reared without animal proteins and AGPs. However, the use of treatment antibiotics continues in a more strategic and targeted programme to ensure bird welfare does not suffer.

**TABLE 1. CHANGES IN BROILER A STARTER DIET COMPOSITION DUE TO INGREDIENT AVAILABILITY**

		NO M+B	NO POULTRY	NO FISH
WHEAT		61.72	63.49	65.62
HIPRO SOYAMEAL		23.67	22.00	21.33
FISHMEAL		2.00	4.00	6.00
MEAT & BONE		2.67	-	-
POULTRY MEAL		3.00	3.00	-
TALLOW		1.87	1.70	-
POULTRY FAT		2.07	2.10	-
MONO DICAL		0.73	1.20	1.30
LIMESTONE		0.80	1.10	1.23
SALT		0.20	0.17	0.10
EXTRUPRO		-	-	-
SOYA OIL		-	-	3.07
VITAMIN PREMIX		0.50	0.50	0.50
METHIONINE DL		0.25	0.23	0.23
LYSINE MONO HCL		0.30	0.30	0.33
THREONINE		0.10	0.10	0.13
SODIUM BICARB		0.12	0.10	0.13
Enzyme NSP		0.01	0.01	0.01
<b><u>CALCULATED ANALYSIS</u></b>				
PROTEIN	%	23.06	22.42	21.67
OIL 'A'	%	5.93	5.69	4.80
FIBRE	%	2.36	2.29	2.28
ASH	%	5.56	5.79	5.95
D:M:	%	88.18	88.09	88.04
TLYSINE	%	1.44	1.44	1.43
DG LYS	%	1.30	1.30	1.30
METH	%	0.60	0.60	0.61
DG METH	%	0.57	0.57	0.58
CALCIUM	%	1.01	1.00	1.00
T:PHOS	%	0.75	0.75	0.76
DG PHOS	%	0.43	0.43	0.43

**TABLE 2. CHANGES IN BROILER A FINISHER DIET COMPOSITION  
DUE TO INGREDIENT AVAILABILITY**

		NO M+B	NO POULTRY	NO FISH
WHEAT		65.84	64.04	63.69
BARLEY		5.00	5.00	5.00
HIPRO SOYAMEAL		15.33	19.00	21.00
MEAT & BONE		5.00	-	-
POULTRY MEAL		3.00	3.00	-
TALLOW		1.13	2.27	-
POULTRY FAT		3.00	3.00	-
MONO DICAL			1.30	1.50
LIMESTONE		0.33	0.93	1.03
FISHMEAL		-	-	1.00
SOYA OIL		-	-	2.33
VEG. FAT BLEND		-	-	3.00
EXTRUPRO		-	-	-
SALT		0.20	0.30	0.27
VITAMIN PREMIX		0.50	0.50	0.50
METHIONINE DL		0.15	0.17	0.15
LYSINE MONO HCL		0.30	0.30	0.30
THREONINE		0.10	0.10	0.13
SODIUM BICARB		0.10	0.08	0.08
Enzyme NSP		0.01	0.01	0.01
<b><u>CALCULATED ANALYSIS</u></b>				
PROTEIN	%	19.80	18.83	18.40
OIL 'A'	%	6.18	6.87	6.71
FIBRE	%	2.40	2.39	2.42
ASH	%	4.43	5.19	5.39
D:M:	%	88.08	88.06	88.09
TLYSINE	%	1.16	1.16	1.14
DG LYS	%	1.04	1.04	1.04
METH	%	0.44	0.45	0.43
DG METH	%	0.41	0.42	0.41
CALCIUM	%	0.85	0.86	0.85
T:PHOS	%	0.63	0.68	0.69
DG PHOS	%	0.37	0.37	0.37

**TABLE 3. CHANGES IN A TURKEY STARTER DIET COMPOSITION DUE TO INGREDIENT AVAILABILITY**

		NO M+B	NO POULTRY	NO FISH
WHEAT		41.96	42.12	40.44
BARLEY		5.00	5.00	5.00
HIPRO SOYAMEAL		37.00	37.00	37.00
FISHMEAL		2.66	3.48	5.24
MEAT & BONE		2.00	-	-
POULTRY MEAL		3.60	3.60	-
TALLOW		1.00	1.00	-
POULTRY FAT		2.38	2.70	-
MONO DICAL		1.94	2.40	2.54
LIMESTONE		1.06	1.30	1.46
SOYA OIL				2.00
EXTRUPRO				5.00
MAIZE GLUTEN 60				1.00
SUNFLOWER				2.00
PEAS				2.00
SALT		0.06	0.06	0.06
VITAMIN PREMIX		0.50	0.50	0.50
METHIONINE DL		0.23	0.23	0.21
LYSINE MONO HCL		0.30	0.30	0.30
THREONINE		0.06	0.06	0.06
SODIUM BICARB		0.17	0.17	0.11
<b><u>CALCULATED ANALYSIS</u></b>				
PROTEIN	%	28.38	27.93	28.14
OIL 'A'	%	5.47	5.66	3.76
FIBRE	%	2.55	2.51	3.01
ASH	%	7.36	7.63	8.00
D:M:	%	88.39	88.38	88.26
TLYSINE	%	1.85	1.85	1.85
DG LYS	%	1.68	1.68	1.69
METH	%	0.67	0.67	0.68
DG METH	%	0.63	0.63	0.64
CALCIUM	%	1.36	1.37	1.37
T:PHOS	%	1.05	1.06	1.09
DG PHOS	%	0.65	0.65	0.65

**TABLE 4. CHANGES IN A TURKEY FINISHER DIET COMPOSITION  
DUE TO INGREDIENT AVAILABILITY**

		NO M+B	NO POULTRY	NO FISH
WHEAT		61.23	57.67	46.23
BARLEY		5.00	5.00	5.00
HIPRO SOYAMEAL		10.60	15.80	14.00
MEAT & BONE		6.00	-	-
POULTRY MEAL		5.00	5.60	-
TALLOW		8.08	9.28	-
POULTRY FAT		3.00	3.00	-
MONO DICAL		0.03	1.54	2.00
LIMESTONE		0.03	0.70	0.90
FULL FAT SOYA		-	-	7.00
EXTRUPRO		-	-	8.00
MAIZE GLUTEN		-	-	1.84
SOYA OIL		-	-	2.00
VEG. FAT BLEND		-	-	7.00
BISCUIT MEAL		-	-	5.00
SALT		0.14	0.26	0.22
VITAMIN PREMIX		0.50	0.50	0.50
METHIONINE DL		0.13	0.30	0.12
LYSINE MONO HCl		0.06	0.02	0.03
THREONINE		0.02	0.18	-
SODIUM BICARB		0.17	0.14	0.15
Enzyme NSP		0.01	0.01	0.01
<b><u>CALCULATED ANALYSIS</u></b>				
PROTEIN	%	18.62	18.27	18.06
OIL 'A'	%	13.30	14.02	13.70
FIBRE	%	2.17	2.08	2.86
ASH	%	4.28	5.15	5.62
D:M:	%	89.00	88.97	89.29
TLYSINE	%	0.92	0.93	0.90
DG LYS	%	0.80	0.80	0.80
METH	%	0.40	0.57	0.40
DG METH	%	0.37	0.54	0.37
CALCIUM	%	0.91	0.91	0.90
T:PHOS	%	0.68	0.74	0.77
DG PHOS	%	0.45	0.45	0.45

## **Nutritional factors that affect leg problems in meat poultry: A Review**

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Skeletal disorders are one of the most prevalent welfare problems in broiler and turkey production; especially those that cause lameness that impair movement or bone breakage during catching and transportation (Julian, 2004; Mench, 2004). Leg problems are also a production problem, and it can be one of the most expensive diseases in terms of output at the farms and carcass condemnations during processing, resource wastage by culled birds, and treatment and prevention costs (Gregory and Wilkins, 1992; Pattison, 1992; Bennett *et al.*, 1999, Whitehead, 2003). Bone fragility and porosity also are correlated with the incidence of bone fragments in deboned meat products, and with discoloration of meat adjacent to bone due to leaching of blood; the product may be less appealing to consumers (Gregory and Wilkins, 1992).

In the USA, the last large survey about leg problems in broilers was done in 1993 (Morris). The results of this survey indicated that leg problems were responsible for 1.1% of broiler mortality and 2.1% of carcass condemnations and downgrades annually, and cost the broiler industry billions of dollars each year (Pattison, 1992). Since, the time of this survey great improvement has been done through out genetic selection, however leg problems continue to be affecting some broiler genetic lines under some growing conditions, but especially affects turkeys (Whitehead, 2003).

Currently, leg problems are a main concern turkey production world-wide. It has been estimated that 2-6% of all turkey flock display some observable signs of skeletal problems, while many more will be affected in a less visible way (Lilburn, 1994). Currently, the incidence of leg abnormalities may exceed 15% in some flocks and up to 5% of the mortality of a flock could be due only to leg problems, which costs the US turkey industry over 150 million per year. Turkeys exhibit more leg problems after 14 weeks of age than younger birds, and the incidence is greatest in winter than in summer seasons (Vaillancourt *et al.*, 1999, 2000; Woodward, 2004). However, the origin of these problems might start even during the embryonic period (Farquharson, 2003; Van der Eerden *et al.*, 2003).

The skeletal system consists of bones, cartilage, ligaments and tendons and accounts for about 20 percent of the body weight of most vertebrates. The syndromes that cause lameness in the birds of a flock can be affecting one or several of these tissues in the same bird or within the flock at the same time (Riddell, 1992; Thorp, 1992; Whitehead *et al.*, 2003; Crespo and Shivaprasad, 2003; Julian, 2004; Mench, 2004; Pines *et al.*, 2005).

In rough order of importance, leg problems include infected hocks (staphylococcal, coliform, viral arthritis, tendinitis), twisted legs (valgus/varus), tibial dyschondroplasia (TD), femoral head necrosis, spondylolisthesis (kinky back), osteomyelitis and pododermatitis (*E. coli*, *Salmonella* or *Staphylococcus*) and slipped tendon. In turkey flocks is most common to find femur spiral fractures (brittle bone), pododermatitis (Clark, 2002) and infectious agents that affect the musculo skeletal system such as *Staphylococcus*, *Mycoplasmas* (*M. meleagridis*, *M. synoviae*, *M. gallisepticum*), and *Ornithobacterium rhinotracheale* (ORT) (Reece, 1992).

Skeletal problems in breeders during lay include hypocalcemia, degenerative joint disease, staphylococcal or viral arthritis/tenosynovitis and keel bursitis (Hocking, 1992). The incidence of other leg defects, such as rotated toes and rickets, have decreased considerably in commercial flocks over the last 20 years due to genetic selection, improved nutrition and litter management.

Lameness is currently measured on-farm by scoring walking ability or willingness of the birds to stand (Mench, 2004). However, in most of the heavy older broilers with abnormal gait, no gross or histological lesions can be observed post-mortem in any of the tissues. The correlation between gait score and specific leg problems, such as TD, varus/valgus deformations, crooked toes, tibial rotation, tendon degeneration and scoliosis, is significant but low (Kestin *et al.*, 1999, Bokkers and Koene, 2000; Sanotra *et al.*, 2001). Part of the lameness problems may be due to conformation of fast-growing high-breast meat yield birds. These birds normally have short legs and large thigh muscle masses, and the development of large breast muscles moves their gravity cranially (Corr *et al.*, 2003a). To compensate for this variations, they walk slowly with their toes pointed outwards, and taking short, wide steps (Corr *et al.*, 2003b), and with aging they become less active (Kestin *et al.*, 2001). Then, solutions to leg problems can not be limited to just improve bone development, strength or density.

Many of the syndromes are poorly understood and multiple factors are involved in their etiology (Rath *et al.*, 2000; Whitehead *et al.*, 2003). Multitude of factors can cause bone development disorders, possibly by several distinct mechanisms, but each results in the occurrence of histological, anatomical and structural similar lesions (Farquharson, 2002; Pines *et al.*, 2005). Possible causes of skeletal defects include infection, malabsorption of nutrients due to enteritis, abnormal bone, cartilage or tendon development, or changes in metabolism as a result of genetic mutations or polymorphisms caused by human selection, or even inadequate nutrition or management, toxins or environmental factors. Litter conditions can play an important role on infectious related problems that can start by a pododermatitis (bumblefoot) and could cause hock or hip joint infectious (Clark *et al.*, 2002; Mayne, 2005).

The control and even treatment of skeletal abnormalities depends on the cause of the problem. If an infectious disease has been confirmed, antibiotics and/or improvements in biosecurity or vaccination programs may help, while nutritional defects may be corrected by attention to the balance diet and micronutrients for specific populations. However, very few skeletal problems are controlled solely by these means. Most of the skeletal disorders currently observed have strong correlation with genetics and management practices, so genetic selection programs and husbandry practices play an important role in their future control (Farquharson, 2002; Whitehead *et al.*, 2003), but nutrition might still solve some of the field cases.

## **Understanding bone development and regulation for better nutrition**

Since bone is the biggest part of the skeletal system, it has been the component of the skeletal system most commonly studied in avian species. Working with a fast grow animal is important to understand the regulation of bone growth. Nevertheless, it is important to remember that ligaments and tendons might have an important role.

**Bone composition, strength and mechanical properties.** Bone is a complex tissue made up of living cells (2%) enmeshed in a mineralized collagenous rich matrix (98%). This matrix is constituted by collagen (80-90%), which in its mature form is formed by pyridinium crosslinks (hydroxylysyl pyridinoline and lysyl pyridinoline), and proteoglycans, lipids, and noncollagenous proteins (osteocalcin, osteonectin and osteopontins) that constitutes 10 to 15% of the organic matrix. The noncollagenous proteins contribute to a variety of functions of bone such as matrix stabilization, calcification, and other metabolic regulatory activities.

The mineral matrix is made up of calcium and phosphorus in the form of hydroxyapatite (60 – 70% of bone weight). The inorganic mineral provides shear strength (stiffness) and resists compression, whereas the organic collagen fibers withstand tension and torsion. Because the mineral matrix is the major component of the extracellular matrix, bone mineral density (BMD) is considered to reflect the status of bone health. High positive correlation ( $r = 0.81$ ) between bone breaking strength and BMD were reported by Frost and Rowland (1991) and Orban *et al.* (1993a). But, the correlation between shear force and BMD was only 0.56 and between ash and shear force 0.67 (Onyango *et al.*, 2003).

Rath *et al.* (1999) observed that bone strength showed positive correlations with its ash content, density, pyridinium crosslinks, and the fluorescence of the matrix. However, the correlation was stronger with both pyridinium crosslinks and the fluorescence of the organic matrix. These results suggest that bone strength is influenced by the content of its collagen crosslinks. Several other researchers have agreed that bone mineralization alone is not always the most related to mechanical properties and breaking strength (Ferretti *et al.*, 1984; Rath *et al.*, 2000; Massé *et al.* 2003)

**Bone growth mechanisms.** The terms osteogenesis and ossification are often used synonymously to indicate the process of bone formation. This process actually starts during the embryonic period and continues throughout life. There are two types of ossification: intramembranous and endochondral. Intramembranous ossification involves the replacement of sheet-like connective tissue membranes with bony tissue and is common in certain flat bones of the skull and some of the irregular bones. Then, their development is not discussed herein.

Endochondral ossification involves the replacement of hyaline cartilage with bony tissue. Most of the bones of the skeleton are formed in this manner. Bones grow in length at the epiphyseal plate by a process that is similar to endochondral ossification. The cartilage in the region of the epiphyseal plate next to the epiphysis continues to grow by mitosis. The chondrocytes, in the region next to the diaphysis, age and degenerate. Osteoblasts move in and ossify the matrix to form bone. This process continues throughout life after hatch until maturity when cartilage growth slows and finally stops. When cartilage growth ceases, the epiphyseal plate completely ossifies so that only a thin epiphyseal line remains and the bones can no longer grow in length (Farquharson, 2003).

The mechanisms of long bone growth are similar across animal species. But, there are, major variations in the growth rate between similar bones of different species, bones of an individual animal and the two growing regions (growth plates) of the same bone. In the domestic fowl, the fastest growing bone tip is the proximal tibia that grows at 0.86 mm/day (Kirkwood *et al.*, 1989). Human femur grows at 0.04, mm/day, and rats and rabbit only at 0.22 and 0.39 mm/day, respectively. The rate of bone growth attributed to a specific growth

plate is determined by an interaction between proliferative kinetics, matrix production in the transverse septa, and hypertrophic chondrocyte enlargement.

The growth plate matrix is formed by collagen type II, which interacts with collagen types IX and XI to form heterotypic fibrils that are distributed throughout the cartilage matrix. During chondrocyte maturation, collagen type II gene expression decreases and the hypertrophic chondrocytes initiate the synthesis of collagen type X-protein unique to this cell type in avian species. Some proteoglycans like aggrecan, and collagenous proteins, such as osteopontin and osteonectin, are also part of this growth plate matrix (Velleman, 2000). All constitute good markers for hypertrophy, which are down regulated in TD lesions (Farquharson, 2002).

Collagen intermolecular cross-links are very important to the mechanical performance of bone (Rath *et al.*, 1999, 2000). The cross-link pattern is determined and influenced by many factors, including the level of lysine hydroxylation, collagen turnover, molecular packing structure, and mineralization; thus, cross-linking is tissue specific rather than collagen type specific.

Terminally differentiated chondrocytes either differentiate into bone cells, proliferating with one daughter chondrocyte dying and the other becoming an osteoblast, or undergo the widely accepted route of programmed cell death. Cartilage mineralization is needed before vascular invasion of the growth plate can occur. This occurs by a series of physico-chemical and biochemical processes. Calcium and phosphorus, and latter hydroxyapatite are deposited in extracellular fluid. Mineralization rate depends on concentration of  $\text{Ca}^{2+}$ ,  $\text{PO}_4^{3-4}$  in the extracellular fluid, pH, and the concentration of proteoglycans that promote mineralization. High concentrations of inorganic phosphate are attributed to the actions of the bone/liver/kidney form of alkaline phosphatase (ALP) (Farquharson and Jefferies, 2000, Farquharson, 2002, 2003). Any failure to accomplish these physiological processes of chondrocyte differentiation/maturation, vascular invasion and matrix mineralization causes TD, and it could be the origin for valgus and varus disorders as well as some breaking strength weaknesses that could cause higher susceptibility to future fractures.

**Hormonal control of bone growth.** Bone growth is under the influence of several endocrine hormones, such as growth hormone (GH), estrogens, Parathyroid Hormone-Related peptide (PTH/PTHrP), Vitamin D, and thyroid hormones ( $\text{T}_3$  and  $\text{T}_4$ ); and autocrine and paracrine factors (produced by bone cells), such as prostaglandins, cytokines, insulin-like growth factor-I (IGF-I), transforming growth factor-B superfamily including BMP, and fibroblast growth factor (FGF). Prostaglandin  $\text{E}_2$  ( $\text{PGE}_2$ ) is known to have diphasic effects on bone formation; at low concentrations it stimulates formation, but a high concentration  $\text{PGE}_2$  inhibits formation (Raisz, 1993, Watkins *et al.*, 2000). Dietary saturated:unsaturated ratios and omega3:omega6 fatty acid balance, vitamin E and other antioxidant vitamins, and biotin can modulate  $\text{PGE}_2$  to obtain the desired effect on bone development (Watkins, 2002, Farquharson, 2003).

The  $\text{T}_3$  and Vitamin D actions are mediated by nuclear receptors ( $\text{T}_3$  and vitamin D receptors -  $\text{T}_3\text{R}$  and VDR) that are structurally homologous members of the steroid/thyroid hormone receptor superfamily, and they function as hormone-inducible transcription factors. The  $\text{T}_3\text{R}$  and VDR recognize similar hormone response element DNA sequences and preferentially bind to DNA as heterodimers with 9-cis-retinoic acid (Pcis-RA) receptors (RXR) (Van der Eerden *et al.*, 2003).

IGF-I stimulates proliferation, whereas  $T_3$  induces hypertrophic differentiation.  $T_4$  is a prohormone that is converted to  $T_3$  by iodothyronine deiodinase 5'-DIO (type 1 to 3), a selenoprotein. GH and glucocorticoids (GC) stimulates this conversion in specific tissues, like chondrocytes located in growth plates. But excessive levels of GH and GC inhibit chondrocyte differentiation in avian species (Monsonogo *et al.*, 1995).

The hormone  $T_3$  in physiological concentrations directly affects cartilage growth and maturation by several means: it stimulates the resting zone cells to differentiate; it is indispensable for chondrocyte hypertrophy (Burch and Lebovitz, 1982); it induces the expression of type II and X collagen and the activity of the differentiation marker ALP; and it is required for vascular invasion of the growth plate and metaphyseal trabecular bone formation (Robson *et al.*, 2002; Van der Eerden *et al.*, 2003). Shen *et al.* (2004) reported that broiler genetic lines susceptible to TD have inadequate gene expression of DIO type 2 in the growth plates and they concluded that this bone disorder is a result of tissue hypothyroidism. Hypothyroidism causes down-regulation of chondrocyte and osteoblastic cells IGF-I, failure of hypertrophic chondrocyte differentiation, absence of collagen-X expression, and increased PTHrP mRNA expression (Robson *et al.*, 2002). Early hypothyroidism in life may result in increased bone density at maturity, but increased fracture rate in adult animals.

***Normal parameters of bone growth in chickens and turkeys.*** Bone development in broiler chickens has been described by Skinner and Waldroup (1995), Rose *et al.* (1996), Leterrier and Constantin (1999); Rath *et al.* (1999), Williams *et al.* (2000, 2004), Yalçın *et al.* (2001), and Torres *et al.* (2005). There are a wide variety of experimental techniques and parameters available for evaluation of bone structure, microstructure, and biomechanics (Turner and Burr, 2001). Some of the parameters used to describe the biomechanical properties of poultry bones are described in Table 1. The status of bone may be poorly described by just one of these properties. For example, a highly mineralized bone (high BMD) will tend to be very stiff, but also brittle, resulting in reduced work to failure and increased risk of fracture. On the other hand, a bone from young animals will tend to be poorly mineralized and weak, but very ductile (large ultimate displacement), resulting in increased work to failure (Turner and Burr, 2001; Carter and Spengler, 2002).

Poultry researchers normally observe differences in bone development due to gender, genetic strain, and growth rates. These differences between males and females are evident after the second week of age with females having lower values. Females generally show lower diaphyseal diameters, which is counterbalanced by modifications in the composition of the matrix and in the porosity of the cortex, leading normally to equal biomechanical characteristics of tibiotarsi in both sexes.

Across broiler strains rapid bone formation occurs between 4 to 18 days and the highest rate of mineralization between 4 to 11 days (Williams *et al.*, 2000). The early rapid bone deposition at the periosteal surface is associated with decreased mineralization, changes in calcium:phosphorus ratios, increased cortical size and porosity, and altered biomechanical properties. The differences observed between strains for bone anatomy and bone mineralization during the rapid growth period around 16 d are not significant at later ages, with the exception of bone volume. Several authors conclude that growth rate, and not the genetic potential, of the fast growing birds is responsible for the rapid periosteal bone deposition and might cause some bone developmental problems (Williams *et al.*, 2004; Whitehead *et al.*, 2004).

**Table 1.** Normal physical, mechanical and biochemical parameters of broiler chicken tibias

Age (days)	Physical and chemical variables			Mechanical variables			
	Trait	Males	Females	Trait	Males	Females	
21	Live body weight (g) <sup>1</sup>	786 ± 121 <sup>a</sup>	679 ± 97 <sup>b</sup>	Porosity (%) <sup>1</sup>	45.0 ± 1.7 <sup>ns</sup>	44.63 ± 1.96 <sup>ns</sup>	
28		1326 ± 212 <sup>a</sup>	1071 ± 170 <sup>b</sup>		40.7 ± 1.8 <sup>ns</sup>	40.42 ± 2.35 <sup>ns</sup>	
35		1559 ± 229 <sup>a</sup>	1287 ± 211 <sup>b</sup>		36.3 ± 2.3 <sup>ns</sup>	34.83 ± 3.03 <sup>ns</sup>	
42		2305 ± 382 <sup>a</sup>	1865 ± 401 <sup>b</sup>		36.3 ± 2.9 <sup>ns</sup>	36.44 ± 2.84 <sup>ns</sup>	
21	Tibia weight (g) <sup>1</sup>	6.9 ± 1.3 <sup>a</sup>	5.65 ± 0.77 <sup>b</sup>	Area (cm <sup>2</sup> ) <sup>1</sup>	0.21 ± 0.03 <sup>a</sup>	0.16 ± 0.02 <sup>b</sup>	
28		11.7 ± 1.4 <sup>a</sup>	8.73 ± 1.16 <sup>b</sup>		0.29 ± 0.05 <sup>a</sup>	0.21 ± 0.03 <sup>b</sup>	
35		14.7 ± 2.0 <sup>a</sup>	11.29 ± 1.63 <sup>b</sup>		0.29 ± 0.05 <sup>a</sup>	0.22 ± 0.03 <sup>b</sup>	
42		20.6 ± 2.9 <sup>a</sup>	15.58 ± 2.12 <sup>b</sup>		0.41 ± 0.07 <sup>a</sup>	0.32 ± 0.09 <sup>b</sup>	
21	Tibia length (mm) <sup>2</sup>	73 ± 0.3 <sup>a</sup>	71 ± 0.2 <sup>b</sup>	Maximum force(N) <sup>1</sup>	171.1 ± 24.9 <sup>a</sup>	132.0 ± 24.7 <sup>b</sup>	
28		87 ± 0.3 <sup>b</sup>	84 ± 0.3 <sup>b</sup>		272.3 ± 49.4 <sup>a</sup>	190.1 ± 28.5 <sup>b</sup>	
35		97 ± 0.3 <sup>a</sup>	94 ± 0.4 <sup>b</sup>		235.9 ± 67.2 <sup>a</sup>	178.9 ± 51.5 <sup>b</sup>	
42		110 ± 1 <sup>a</sup>	103 ± 13 <sup>b</sup>		296.5 ± 61.4 <sup>a</sup>	226.8 ± 69.2 <sup>b</sup>	
49		118 ± 1 <sup>a</sup>	114 ± 1 <sup>b</sup>	<b>Load at break (kg/mm<sup>2</sup>)<sup>4</sup></b>			
56		126 ± 1 <sup>a</sup>	121 ± 1 <sup>b</sup>	<b>26.66 ± 4.89      20.96 ± 1.82</b>			
21	Tibia width (mm) <sup>2</sup>	4.9 ± 0.2	4.5 ± 0.1	Flexion resistance (MPa) <sup>1</sup>	100. ± 15.3 <sup>ns</sup>	105.6 ± 17.4 <sup>ns</sup>	
28		5.9 ± 0.2	5.6 ± 0.2		107.6 ± 22 <sup>ns</sup>	118.4 ± 15.0 <sup>ns</sup>	
35		7.1 ± 0.2	6.6 ± 0.2		93.9 ± 16.6	102.2 ± 21.9	
42		8.1 ± 0.2	7.0 ± 0.3		95.7 ± 20.3 <sup>ns</sup>	105.9 ± 24.6 <sup>ns</sup>	
49		8.6 ± 0.2	7.6 ± 0.2				
56		9.5 ± 0.2	7.8 ± 0.3				
21	Density (g/cm <sup>3</sup> ) <sup>1</sup>	1.04 ± 0.1 <sup>ns</sup>	1.08 ± 0.07 <sup>ns</sup>	Inertia x 10 <sup>-10</sup> (m <sup>4</sup> ) <sup>1</sup>	5.19 ± 1.38 <sup>a</sup>	3.53 ± 1.05 <sup>b</sup>	
28		1.12 ± 0.1 <sup>ns</sup>	1.16 ± 0.09 <sup>ns</sup>		10.82 ± 4.32 <sup>a</sup>	5.70 ± 1.44 <sup>b</sup>	
35		1.25 ± 0.1 <sup>ns</sup>	1.30 ± 0.10 <sup>ns</sup>		12.81 ± 3.72 <sup>a</sup>	7.96 ± 3.11 <sup>b</sup>	
42		1.14 ± 0.1 <sup>ns</sup>	1.17 ± 0.11 <sup>ns</sup>		21.30 ± 7.84 <sup>a</sup>	13.90 ± 7.91 <sup>b</sup>	
21	Tibia Ash <sup>*</sup> (%) <sup>3,4‡</sup>	48.9 <sup>3</sup>	62 <sup>3</sup>	<b>Stress<sup>49a</sup> (kg/mm<sup>2</sup>)</b>	<b>1.76 ± 0.22<sup>4</sup></b>	<b>1.97 ± 0.40<sup>4</sup></b>	
28		63 <sup>3</sup>	63 <sup>3</sup>				
35		64.98 ± 0.8 <sup>4</sup>	64.03 ± 1.1 <sup>4</sup>	<b>Strain<sup>49a</sup> (kg/mm<sup>2</sup>)</b>	<b>0.16 ± 0.02<sup>4</sup></b>	<b>0.21 ± 0.02<sup>4</sup></b>	
49							
21	Tibia Ash (%) <sup>2‡</sup>	41.86 ± 0.80	43.62 ± 0.62	Young's module* (GPa) <sup>1</sup>	3.05 ± 0.50 <sup>b</sup>	3.75 ± 0.60 <sup>a</sup>	
28		41.93 ± 0.45	43.59 ± 0.42		3.14 ± 0.89 <sup>b</sup>	4.69 ± 0.69 <sup>a</sup>	
35		42.43 ± 0.34	44.13 ± 0.69		3.59 ± 0.73	5.01 ± 1.14	
42		42.86 ± 0.78	44.35 ± 0.54	3.70 ± 0.79 <sup>b</sup>	5.105 ± 1.52 <sup>a</sup>		
49		43.43 ± 0.43	43.83 ± 0.66	<b>Modulus (kg/mm<sup>2</sup>)<sup>4</sup></b>	<b>26.60 ± 6.69</b>	<b>25.62 ± 5.34</b>	
56		43.73 ± 0.61	45.49 ± 0.53				
21	Tibia Calcium (%) <sup>2</sup>	14.31 ± 0.52	14.92 ± 0.17	<b>Matrix biochemical constituents at 49 d<sup>4</sup></b>			
28		14.30 ± 0.19	14.49 ± 0.33	----- (µg/mg of tibia bone) -----			
35		14.36 ± 0.20	14.93 ± 0.59	Collagen	225.51 ± 4.40	221.56 ± 4.88	
42		15.29 ± 0.30	17.70 ± 0.99	Proteoglycan	1.56 ± 0.55	1.40 ± 0.07	
49		15.45 ± 0.16	18.30 ± 0.25	<b>Bone collagen composition at 49 d<sup>4</sup></b>			
56		14.78 ± 0.59	20.08 ± 0.49	----- (µg/mg of collagen) -----			
21	Tibia phosphorus (%) <sup>2</sup>	7.25 ± 0.24	7.48 ± 0.21	HP	0.026 ± 0.006	0.026 ± 0.006	
28		7.68 ± 0.28	7.97 ± 0.15		LP	0.016 ± 0.003	0.018 ± 0.006
35		7.56 ± 0.14	7.60 ± 0.28		Total	0.042 ± 0.003	0.044 ± 0.004
42		7.72 ± 0.22	9.12 ± 0.57	pyridinolines	Collagenase extractable fluorescence (AFU/mg bone)	1.96 ± 0.16	2.31 ± 0.17
49		7.43 ± 0.15	9.88 ± 0.74				
56		8.10 ± 0.21	9.94 ± 0.71				
21	Ca:P ratio (M)	1.97	1.99	Collagenase resistant matrix (%)	14.77 ± 2.30	21.08 ± 2.88	
28		1.86	1.82				
35		1.90	1.96				
42		1.98	1.94				
49		2.40	2.32				
56		1.82	2.02				

<sup>ab</sup>Means with the same superscript do not differ significantly by Tukey's test (P>0.05); <sup>ns</sup> Non-significant Sources: <sup>1</sup>Torres *et al.*, 2005; <sup>2</sup>Adapted from Skinner and Waldroup, 1995; <sup>3</sup>Boskey, 2002; <sup>4</sup>Rath *et al.*, 1999 - HP = hydroxylysylpyridinoline; LP = lysylpyridinoline; AFU = arbitrary fluorescence unit. \* Elasticity modulus. <sup>1</sup>Density (mm of aluminum equivalents) at 21 d 2.17 ± 0.03 (Soares da Silva *et al.*, 2003). <sup>\*</sup> Bones ashed at 750 °C for 22 h without fat extraction. <sup>‡</sup> Tibia ash after removing the fat by 20 hr extraction in hot alcohol and 20 hr in ether and ashed at 550 °C.

The general form of each bone is genetically determined, but the specific architecture that is critical for structural bone competence develops and persists only in response to continued exposure to loading. Loading is osteogenic, but this stimulus appears to saturate after a few daily loading cycles (Lanyon, 1992). Recently, Reich *et al.* (2005) demonstrated in an elegant study with broilers that moderate (10%) increments in body weight (by weight loading bags) at an early age and for short periods of time (2 – 4 days) accelerates cartilage resorption, increases osteopontin and matrix metalloproteinases expression, that enhances early mineralization resulting in narrower growth plates and shorter bones. This can explain differences in bone length and structure of chickens with different early body weight gains.

Some bone morphology and biomechanical parameters are commonly overlooked in poultry bone research, but they can explain some of the bone disorders observed in the field. For example, the cross-sectional moment of inertia (CSMI), which is a parameter of morphology, gives an idea about the distribution of bone material around a given axis in the cortical area. CSMI is calculated by the diameters of bone cross section and cortical thickness. The collagen fiber orientation is another parameter that is important according to the type of strain applied to the bone, tension or compression. Longitudinal orientation is better in tension and transversal in compression. Collagen fiber orientation is evaluated based on birefringence of bone under polarized light or circularly polarized filters (Turner and Burr, 2001; Carter and Spengler, 2002).

Mechanical evaluations, such as the three-point loading/bending tests, are common among poultry literature. However, other important evaluations like tensile testing, compressive testing, four point loading tests, torsion testing, pure shear tests and fatigue testing are not commonly evaluated and they can give important characteristics of bone integrity depending on the problem to be evaluated (Turner and Burr, 2001; Carter and Spengler, 2002).

We have noticed that there is high variability in the data published related to bone development due to the methodology of analyses, which was also discussed by Rath *et al.* (2000). Fresh bone gives better bone breaking strength correlated to the other bone parameters than dry or fat-free dry preparation (Kim *et al.*, 2004). At the moment of evaluating bone properties, it is important to keep the samples hydrated with a physiological saline solution, and at physiological temperature (40 – 41 °C), otherwise the mechanical properties change dramatically. Failure to control these two factors can cause variabilities in tensile strength (31%), toughness (55%) and fatigue (100%) in the experimental values. The rate at which loading is applied during biomechanical testing of bone affects the measured stiffness and strength. If one is trying to simulate physiological conditions, the strain rate should be between 0.01 and 0.08/s, which is within the range of strain rates that occurs *in vivo* (Turner and Burr, 2001).

Bonser and Casinos (2003) used microhardness testing as a probe for fine-scale regional variation in the mechanical performance of bones and present data showing the extent of regional variation in the femora and humeri of 7-week-old broiler birds. Ash content of dry bone was broadly correlated with microhardness, although there is some evidence that the relationship linking the two differs between the femur and the humerus. Regional variations in the properties of avian bones are widely overlooked in the literature. Awareness of them is vital and existing measures of bone 'strength' may be misleading if local variation in

properties is not taken into account when exploring the effects of nutrition and husbandry practices on bone mechanical performance.

***Genetic background, endocrine system and nutrition in modern poultry.*** Lameness, leg and skeletal problem incidence definitely can be diminished by appropriate multi-trait selection with a continued improvement in growth rate (Whitehead *et al.*, 2003). Some commercial chicken strains appear to have a genetic predisposition to skeletal abnormalities when management practices and their accelerated growth rates increases the amount of defects in their bones (Kestin *et al.*, 1999, 2001; Whitehead, 2003; Mench, 2004). On the other hand, Hafez *et al.* (2004) investigated five commercial meat turkey lines (Kelly Bronze, Nicholas 300, BUT 9, Nicholas 700, and BUT-Big 6) regarding leg disorders. Under commercial rearing and feeding conditions these turkey lines are characterized by different body weight development. The obtained results revealed that there was no correlation between turkey lines and the incidence of TD, fractures, and arthritis.

Modern broilers and turkeys have been genetically selected to grow faster, have higher feed intake and better feed conversion, and yield more breast meat, but at the same time some modifications in the endocrine mechanisms have occurred especially in thyroid and GH hormone production, elimination, tissue utilization and peripheral conversion (Gonzales *et al.*, 1999; Rahimi, 2005). Thyroxine (T<sub>4</sub>) and triiodothyronine (T<sub>3</sub>) has great influence over avian bone development (Bonser *et al.*, 2004), and even some common bone disorders, such as TD in broilers have recently been correlated to tissue-specific hypothyroidism (Shen *et al.*, 2004). Deficiency in the thyroid hormones and elevated corticosterone play a key deleterious role in the development of other metabolic disorders typical of fast-growing birds like the ascites syndrome (Luger *et al.*, 2002).

A more pulsatile GH secretion, common in commercial strains of poultry, can inhibit differentiation in avian growth-plate chondrocytes, thereby sustaining their proliferative state and maintaining their sensitivity to growth factors such as EGF (Monsonego *et al.*, 1995). Consequently, it is not surprising that chickens affected with TD have elevated levels of plasma GH (Vasilatos-Younken and Leach, 1986).

Additionally, several minerals and trace elements are essential for normal thyroid hormone metabolism, namely iodine, iron, selenium, and zinc. Coexisting deficiencies of these elements can impair thyroid function (Zimmermann and Kohrle, 2002). In the same way, the metabolism of zinc, copper, magnesium, calcium, manganese, and selenium, (Aihara *et al.*, 1984; Simsek *et al.*, 1997), as well as vitamin A (Aktuna *et al.*, 1993) is abnormal in thyroid diseases. Altered zinc turnover also may be also involved in depressed thyroid-thymus axis efficiency. Zinc turnover is under the control of zinc-bound metallothioneins (Zn-MTs) synthesis. Thyroid hormones, corticosterone and nutritional zinc affect Zn-MT induction (Mocchegiani *et al.*, 2002) and thyroid hormones mediate changes in membrane fluidity that might play an important role in modulating Zn<sup>2+</sup> transport activity of intestinal and renal brush-border membranes controlling its absorption and reabsorption (Prasad *et al.*, 1999). The T<sub>3</sub> receptor is thought to require zinc to adopt its biologically active conformation. Some of the effects of zinc deficiency, therefore, may be due to loss of zinc from the T<sub>3</sub> receptor and impairment of T<sub>3</sub> action (Freake *et al.*, 2001).

Thyroid function can also be altered by management practices (Yahav *et al.*, 2000), incubation conditions during the embryonic period (Christensen *et al.*, 2002; Decupere,

2005), nutritional levels, brooding temperatures and feeding programs ((Noy *et al.*, 2001; Proudman and Siopes, 2005). Plasma T<sub>3</sub> concentrations, which may mediate some of the intestinal effects of feed deprivation, were depressed in poult without access to feed (Noy *et al.*, 2001). High temperatures reduce T<sub>3</sub> levels and exposition to low temperatures increase T<sub>3</sub> (Haddad and Mashaly, 1989). Plasma T<sub>3</sub> is positively and significantly correlated with food intake (Yahav, 2000) Even, maternal thyroid function can affect thyroid function in the progeny and this can be modified by iodine supplementation (Christensen and Davis, 2001). Christensen *et al.* (1996) observed in turkey hens that increased (P < 0.05) T<sub>4</sub> was evidenced in embryos from youngest hens, whereas increased (P < 0.05) T<sub>3</sub> activity was evident in embryos from hens of older ages. Similar effects have been observed in broilers, affecting bone weight, ash content and bone volume on day of hatch, but the extent of this effect also depended on the strain (Yalçın *et al.*, 2001).

These evidences indicate that commercial broilers and turkeys can be exposed to constant variations in thyroid status that can affect bone development. It is interesting to note that the proven successful nutritional methods to resolve bone problems are also similar to the ones observed to improve developmental problems in hypothyroid animals.

## **Nutrition and skeletal problems in meat poultry**

Skeletal problems in poultry have been associated with the dietary levels of vitamins like Vitamins D, A, C and K and most B-vitamins, but especially pyridoxine and folic acid; minerals, such as calcium (Ca), phosphorus (P), sodium (Na), chloride (Cl), zinc (Zn), selenium (Se), copper (Cu), manganese (Mn); protein levels or amino acids, such as methionine, cystine, cysteine and the metabolite homocysteine; fatty acids; and total feed consumption. Bone development is also affected by metabolic acidosis induced by dietary electrolytes, toxic minerals like cadmium that contaminate other mineral sources, and mycotoxins coming by fungi proliferation on grains stored for long periods of time.

Some of the leg disorders observed in the field could be caused more frequently by feed processing factors than for inadequate feed formulation. Situations such as high variability on feed ingredient composition, feed mixing problems, fat and oil oxidization and uncertainty of nutrient digestibility and bioavailability are common in commercial feed mills. Improve feed quality control is essential to solve any of these problems.

### ***Vitamins***

***Vitamin D.*** Vitamin D is composed for a group of molecules similar to steroids called secosteroids, which include cholecalciferol (Vitamin D<sub>3</sub>), ergosterol (Vitamin D<sub>2</sub>), 24,25 (OH)<sub>2</sub>D<sub>3</sub>, and their photoisomers. These compounds play an important role in Ca homeostasis, regulation of cell differentiation (genomic responses) in several tissues such as the immune system, skin and different bone cell populations (Whitehead, 2002; Holick, 2004; Dusso *et al.*, 2005).

Vitamin D action is mediated throughout an interaction with the cellular nuclear receptors (VDR). In this way this pro-hormone regulate gene transcription with synthesis of typical proteins, but there are other effects of this vitamin that may result from non-genomic mechanisms, which are membrane-associated processes (Lu *et al.*, 2000; Dusso *et al.*, 2005). The genomic effects could last hours and involve in the bone genes for the bone protein

osteocalcin as well as the matrix GLA protein, and osteoclast generation. While the non-genomic effects last only minutes and involve  $\text{Ca}^{2+}$  channels and membrane receptors.

Vitamin  $\text{D}_3$  deficiency results in hypocalcemia and hypophosphatemia. Vitamin  $\text{D}_3$  is absorbed into the blood and stored in the liver, and so circulating levels of  $\text{D}_3$  are usually low. Circulating 25 (OH) $\text{D}_3$  levels in broilers should be at least 5 ng/ml (Goff, 1992). In vitamin  $\text{D}_3$  deficient chicks, bone resorption is inhibited after about 7 d, while the formation of osteoid continues, resulting in an increased total bone mass.

The biopotency of  $\text{D}_3$  sources is still controversial. In avian species, vitamin  $\text{D}_3$  is 30 to 40 more potent than vitamin  $\text{D}_2$ . 1,25(OH) $_2\text{D}_3$  is able to prevent rickets with five times the potency of vitamin  $\text{D}_3$ . Ferretti *et al.* (1984) concluded that in order of potency to promote bone strength, the sources can be ranked 1,25(OH) $_2\text{D}_3$  > 25-OH- $\text{D}_3$  > VIT- $\text{D}_3$  (Leeson *et al.*, 1995).

Fritts and Waldroup (2003) evaluated cholecalciferol (VIT- $\text{D}_3$ ) and 25-hydroxycholecalciferol (25-OH- $\text{D}_3$ ) in doses to provide 125, 250, 500, 1,000, 2,000, or 4,000 IU/kg of vitamin D activity in a nutritionally complete corn-soybean meal diets. They observed that the incidence and severity of TD was significantly lower for birds fed 25-OH- $\text{D}_3$  and was reduced by increasing levels of vitamin D, regardless of source. Results of this study show that 25-OH- $\text{D}_3$  is more metabolically potent on a per unit basis than vitamin  $\text{D}_3$  for support of body weight, tibia ash, and reduction in incidence and severity of TD. The differences were observed primarily at lower levels of vitamin D. At typical industry levels, few differences were observed between the two sources. The utilization of 25-OH- $\text{D}_3$  may allow for supplementation with lower levels or may provide a greater margin of safety, especially in enteritis problems because 25-OH- $\text{D}_3$  can be absorbed better than Vitamin  $\text{D}_3$ . Ledwaba and Roberson (2003) reported increased body weights and tibia ash and decreased TD incidence and severity as the dietary 25-OH- $\text{D}_3$ , but this response was dependent upon the calcium level.

The requirement for cholecalciferol ( $\text{D}_3$ ) varies according to the parameter evaluated and according to the dietary level of Ca or P (Leeson *et al.*, 1995; Whitehead *et al.*, 2004). High levels of vitamin  $\text{D}_3$  have failed to prevent rickets or TD incidence (Edwards 1990, 2003; Atencio *et al.* 2005), even there is little response to extra supplementation of vitamin  $\text{D}_3$  when Ca and P levels are adequate.

Genetics always plays a role in the responses to any nutrient supplementation in bone development as was discussed by Edwards (2000). The supplementation with high levels of three derivatives of Vitamin D ((25-(OH) $\text{D}_3$ , 1,25-(OH) $_2\text{D}_3$ , and 24R,25-(OH) $_2\text{D}_3$ ), or ultraviolet light can decrease incidence of TD in lines selected for low incidence of TD, while none of these treatments is able to reduce the incidence in strains with high incidence. It seems that this effect is mainly linked to fail in chondrocyte differentiation and expression of VDR (Berry *et al.*, 1996).

The amount of vitamin  $\text{D}_3$  stored in the yolk sac is very important for future growth and bone development. Atencio *et al.* (2005) observed chicks hatched from eggs laid by hens fed 2,000 or 4,000 IU of  $\text{D}_3$ /kg as the maximum level of vitamin  $\text{D}_3$  had the highest body weight gains and bone characteristics. Chicks fed 3,200 IU had the highest body weight and tibia ash and the lowest TD and Ca rickets incidences. The commercial levels used by the industry

in the US are commonly 9 times higher than the NRC recommendations for broilers (Table 2), and at least 3 times higher for turkeys (Table 3).

Exposure to ultraviolet light (285-365 nm) for short periods of time (11 to 45 minutes) is enough to produce the equivalent to 20 to 40 µg/kg of vitamin D<sub>3</sub> in the feed, and the 1,25-(OH)<sub>2</sub>D<sub>3</sub> or the photoisomers generated are able to reduce the incidence and severity of TD under adequate and inadequate dietary levels of Ca, P or vitamin D<sub>3</sub>. The same effect could not be observed even by increasing in 10 times the recommended (NRC, 1994) dietary level of Vit. D<sub>3</sub> (Edwards, 2000; 2003). The exposure to UV light is more effective when done at first day of age for 30 minutes than latter on in life and for longer periods of time (Edwards, 2003). Lewis *et al.* (2000) did not observe significant improvements in leg integrity or performance in male turkeys reared in environments supplemented with UV light, but with white light intensities higher than 10 lux.

**Other Lipid-soluble Vitamins and Bone Development.** Vitamin D is a lipid-soluble vitamin and competes for absorption with other vitamins like A and E. Oxidized fats degrade activity of vitamin D and all fat soluble vitamins. Very high dietary levels of vitamin A. (45,000 IU/kg) and E (10,000 IU/Kg) (Tables 2 and 3) may increase the requirement for cholecalciferol (Aburto and Britton, 1998; Aburto *et al.*, 1998); however, moderate levels of vitamin E (150 IU/kg) do not exacerbate cholecalciferol deficiency in young broilers (Bartov, 1997).

Vitamin E also affects bone formation in poultry. Supplemental vitamin E increased the bone formation rate in broiler chicks fed diets containing varying levels of unsaturated fatty acids (Xu *et al.*, 1994b). The status influences the amount of free radicals in plasma and liver of broilers. This is important because the mineralized region of growth cartilage may have limited capacity to handle oxidized lipids, since superoxide dismutase and catalase activities are low in this region of cartilage (Matsumoto *et al.*, 1991), and the osteoclastic bone resorption may be enhanced by free radicals (Garret *et al.*, 1990).

**Table 2.** Average vitamin fortification levels (*times of NRC 1994 recommendations*) for commercial market broilers according to feeding phase.

Vitamin	Units	NRC (1994)	% NRC supply by corn-soy <sup>†</sup>	Times NRC		
				Starter	Grower	Finisher
Retinol, A	IU/kg	1500	97	5.4	4.6	3.9
Cholecalciferol, D <sub>3</sub>	IU/kg	200	-	12.8	10.8	9.5
Tocopherol, E	mg/kg	10	121	2.2	1.6	1.4
Menadione, K <sub>3</sub>	mg/kg	0.5	80	3.4	2.9	2.6
<b>Thiamine, B<sub>1</sub>*</b>	<b>mg/kg</b>	<b>1.8</b>	<b>194</b>	<b>0.9</b>	<b>0.8</b>	<b>0.7</b>
Riboflavin, B <sub>2</sub>	mg/kg	3.6 – 3	50	1.9	1.6	1.7
<b>Pyridoxine, B<sub>6</sub>*</b>	<b>mg/kg</b>	<b>3.5 - 3</b>	<b>142</b>	<b>0.7</b>	<b>0.6</b>	<b>0.6</b>
Cyanocobalamin, B <sub>12</sub>	µg/kg	10 – 7	0	1.3	1.1	1.3
Niacin, B <sub>3</sub>	mg/kg	35 - 25	57	1.2	1.2	1.2
Panhotenic acid, B <sub>5</sub>	mg/kg	10	80	1.1	1.0	<b>0.9</b>
Folic acid	mg/kg	0.55 – 0.5	72	1.5	1.3	1.2
<b>Biotin, H*</b>	<b>mg/kg</b>	<b>0.15 – 0.12</b>	<b>120</b>	<b>0.6</b>	<b>0.5</b>	<b>0.6</b>

**Source:** Adapted from **BASF, 1998.** \*Vitamins that are fortified below NRC recommended levels. <sup>†</sup>Low bioavailability, Leeson *et al.*, 1995.

Vitamin A deficiency may not only act directly on the growth plate, but may also indirectly affect growth by systemic mechanisms. For example, retinoids appear to be required for GH secretion, and for thyroid hormone secretion and action (De Luca *et al.*, 2000). Dietary vitamin A within the range 2 to 4.5 mg retinol/kg (6600 - 15000 UI/kg) did not show any interaction with vitamin D<sub>3</sub> status at either age (Whitehead *et al.*, 2004).

Zhang *et al.*, (2003) concluded that to gain optimum bone quality and broiler performance, the concentration of vitamin K should be 8 mg/kg, 2 mg/kg, and 2 mg/kg, for the starter, grower and finisher phases, respectively. Furthermore, they showed that the starter period is an important phase for improving bone quality. In addition, this study validated the mechanism of vitamin K effects on bone quality. Vitamin K boosts the carboxylation of osteocalcin and decreases the concentration of serum under-carboxylated osteocalcin enhancing hydroxyapatite binding capacity of serum osteocalcin and improving bone quality.

**Table 3.** Average vitamin fortification levels (*times of NRC 1994 recommendations*) for commercial market turkeys according to feeding phase.

Vitamin	Units	NRC (1994)	Times NRC		
			Starter	Grower	Finisher
Retinol, A	IU/kg	4000	2.9	2.1	1.4
Cholecalciferol, D <sub>3</sub>	IU/kg	900	5.7	3.9	2.6
Tocopherol, E	mg/kg	12 – 10	4.6	2.4	1.1
Menadione, K <sub>3</sub>	mg/kg	1 – 0.8	2.4	2.1	2.1
<b>Thiamine, B<sub>1</sub>*</b>	<b>mg/kg</b>	<b>2</b>	1.4	<b>0.8</b>	<b>0.4</b>
Riboflavin, B <sub>2</sub>	mg/kg	4 – 3	2.6	2.2	1.7
<b>Pyridoxine, B<sub>6</sub>*</b>	<b>mg/kg</b>	<b>4.5 - 3.5</b>	1.04	<b>0.7</b>	<b>0.5</b>
Cyanocobalamin, B <sub>12</sub>	µg/kg	3	6.1	4.2	2.6
Niacin, B <sub>3</sub>	mg/kg	70 – 50	1.3	1.3	<b>0.9</b>
Panthenic acid, B <sub>5</sub>	mg/kg	11 – 9	1.9	1.6	1.2
Folic acid	mg/kg	1 – 0.8	2.1	1.6	1.0
<b>Biotin, H*</b>	<b>mg/kg</b>	<b>0.2 – 0.13</b>	1.1	<b>0.7</b>	<b>0.6</b>

**Source:** Adapted from **BASF, 1998.** \*Vitamins that are fortified below NRC recommended levels

**Vitamin C.** The benefits of supplementing Ascorbic Acid (AA) to prevent leg disorders and cartilage problems in broilers are highly variable. The dietary addition of AA has been observed to increase duodenal Ca-binding protein and plasma 1,25 (OH)<sub>2</sub>D<sub>3</sub>, as evidence of increased 1-hydroxylase activity (Weiser *et al.*, 1988) and AA co-induced with Vitamin D<sub>3</sub> chondrocyte differentiation and mineralization in avian species (Leach and Rosselot, 1992). The last effect could be due to increase in number of VDR (Farquharson *et al.*, 1998). Petek *et al.* (2005) observed reduction in incidence and severity of TD and improvement of the cortical thickness of tibiotarsus by supplementation (150 mg/l) of AA in water, and application of intermittent lighting (12L:3(1L:3D)) programs in broilers.

On the other hand, dietary supplementation at even higher levels of AA has not prevented TD in broilers (Leach and Burdette, 1985; Edwards, 1989b). No clear evidence of interaction has been observed between AA and Vitamin D<sub>3</sub> supplementation to improve bone developmental parameters or TD incidence (Edwards, 1989b; Roberson and Edwards, 1994).

Some benefit on reducing TD was observed when AA was supplemented to diets with only 2 µg/kg of 1,25-(OH)<sub>2</sub>D<sub>3</sub> (Whitehead *et al.*, 1994). Using even high levels (250, 500 or 1,000 mg/Kg of diet) of AA in diets devoid or with only 10 µg/kg of 1,25-(OH)<sub>2</sub>D<sub>3</sub> has not shown to improve TD even under low-Ca, high-P diets (Rennie and Whitehead, 1996).

This high variability in the response to AA supplementation could be due to the genetic variability of the broiler genetic strains used and the level of stress of those chickens during the experiment and the degree of stress during the experiment. The AA supplementation has been clearly linked to modulation of GC, such as corticosterone under heat stress conditions (Mahmoud *et al.*, 2004) and many other multiple stresses (McKee and Harrison, 1995). Ascorbic Acid improves the performance of chicks with experimentally induced hypothyroidism (Takashi *et al.*, 1991). Orban *et al.* (1993b) observed increments in plasma ionic Ca in AA-treated birds, femur strength improvement by 16% in birds fed 2,000 ppm of AA, but other bone characteristics were not affected.

Deficiencies of all **water-soluble vitamins** play important roles in skeletal disorders (Leeson *et al.*, 1995). Although a common corn-soybean meal (Tables 2 and 3) diet may contain enough of these vitamins, it has been proven that they are not sufficiently bioavailable for bone development of fast growing chickens. Sufficient vitamin fortification of diets with synthetic sources is necessary. Vitamin premixes are commonly not formulated to supply the minimum NRC (1994) recommended levels for thiamine, pyridoxine and biotin (Tables 2 and 3, BASF, 1998). Marginal **pyridoxine** deficiencies are involved in hyperhomocysteinemia and reduced activity of lysyl oxidase fundamental for elastin and collagen cross linking. Recent data suggest that homocysteine, folate, vitamins B<sub>6</sub> and B<sub>12</sub> affect bone metabolism, bone quality and fracture risk in humans. **Biotin** dietary levels are associated with pododermatitis in turkeys (Clark *et al.*, 2002; Hafez *et al.*, 2004, Mayne, 2005).

## Minerals

**Calcium and phosphorus.** Almost 99% of body Ca is contained in the skeleton, however is necessary to maintain a constant 2.5 mM or 10 mg/100 ml (non-laying birds) concentration of Ca<sup>2+</sup> in plasma and extracellular fluids. Variations in the molar Ca:P ratio of bone is likely to cause alterations in the bone mineral crystal structure and consequently in the mechanical competence (Thorp and Waddington, 1997). In commercial poultry diets, Ca does not seem to be a problem and current recommended levels are sufficient broiler starter diets, and even could be in excess in grower diets to achieve maximum growth and adequate bone development (Driver *et al.*, 2005).

The source of P and its availability affects bone strength (Hemme *et al.*, 2005). Presently, P requirements are based on consumption of nonphytate P (NPP), which does not account for the fact that NPP may not be completely available and that phytate P can be partially utilized to fulfill phosphorus requirements. Phosphorus retention values for feed ingredients, accounting for NPP and phytate P and total retainable phosphorus requirements, are needed to formulate diets that meet the P requirements for bone development of poultry without causing excessive amounts of P in poultry excreta (Leske and Coon, 2002).

Leske and Coon (2002) determined the retention of the P from different P. The maximum retentions of total P, NPP, and phosphorus from mono calcium phosphate (MCP) for the basal-MCP test diets were 67.6, 80.2, and 98%, respectively. The maximum retention of

dietary retainable P occurred with a 2:1 ratio of 0.48% Ca and 0.24% retainable P. The retainable P intakes for 10-to-15-d-old broilers required to provide a steady physiological state was 108 mg/d, as determined by two-line regression analysis. Retainable P requirements based on segmented line regression analysis using bone strength measurements for 0-to-3-wk-old chicks and 3-to-6-wk-old broilers were 0.39 and 0.30%, respectively. This indicates that current commercial diets might have enough P for bone development. Even, it had been suggested that maintaining a 2:1 Ca:NPP ratio during the 42 – 49 period enabled the removal of supplemental dicalcium phosphate with minimal effect of tibia strength or performance (Skinner and Waldroup, 1992). Chen and Moran (1992) confirmed no effects on live performance whenever the dicalcium phosphate was omitted (Ca:Total P = 1.6), however they observed an increased incidence of carcass defects after automated on-line processing.

Adequate levels of P and supplementation of phytase avoid tibia dischondroplasia (Qian *et al.*, 1996; Scheideler and Ferket, 2000). Sohail and Roland (1999) showed that phytase (300 FTU/kg) had greater influence on bone mineral content, bone density, bone breaking strength, and livability in broilers fed 0.225% NPP than in broilers fed 0.325% NPP. In diets containing marginal to deficient levels of either NPP or Ca or both, the addition of microbial phytase at 300 to 600 FTU/kg feed prevents P deficiency symptoms. Increasing phytase levels from 600 FTU/kg of feed provided no additional benefit. However, if dietary P is reduced relying in the phytase activity, and the mix uniformity of phytase is very bad (CV 103%) the bone ash and breaking strength, and Ca and P retention could be decreased (Johnston and Southern, 2000).

**Zinc.** Zinc is necessary for chondrocyte proliferation and differentiation. Proliferating chondrocytes may have a high requirement for zinc, VDR contain two zinc fingers (Zhongjian *et al.*, 2000). Supplementation of serum-free primary cultures of avian growth plate chondrocytes with 10-100  $\mu\text{M}$  Zn resulted in an increase in cell protein and greatly increased alkaline phosphatase (AP) activity (Litchfield *et al.*, 1998).

A short-term dietary zinc deficiency inhibits chondrocyte proliferation, differentiation, and induces cell apoptosis in the epiphyseal growth plate of young chickens (Wang *et al.*, 2002). The bioavailability of the microminerals is important and varies according to the source (Cao *et al.*, 2000), and the levels deposited in bone increase with the dietary level offered. Organic or chelated forms have been proven to be more efficient to support performance and health, despite of the level of inorganic source used (Kidd *et al.*, 1994a, b).

Congenital rickets observed during the first few days after hatch is usually linked to maternal nutrition or to problems of mineral absorption during the hatching process. Kidd *et al.* (1992) reported that the progeny of breeder hens fed diets supplemented with zinc-methionine had greater tibia bone ash content. Supplementation of 20 and 40 ppm of zinc and manganese as methionine chelate forms significantly reduced the incidence of shaky leg and angular defects in half in comparison to inorganic sulfate form (Ferket *et al.*, 1992).

**Selenium.** Selenium is involved in several metabolic processes such in transulfatation to form cysteine from methionine and in the deionization of thyroid hormones from T<sub>4</sub> to T<sub>3</sub> by 3,5,3' –triiodotironina. These processes are necessary for chondrocyte maturation. Iodothyronine deiodinases are selenoproteins contributing to systemic or local thyroid hormone homeostasis (Köhrle *et al.*, 2005). Selenoproteins are involved in bone metabolism as well as functions of the endocrine pancreas and adrenal glands. Jianhua *et al.* (2000)

showed that increasing dietary levels of Selenium increased 5' deiodinase activity and concentrations of T<sub>3</sub>, with an improvement in live performance.

Moreno-Reyes *et al.* (2001) investigated selenium deficiency on bone metabolism in growing male rats fed a selenium-deficient diet for two generations (Se-). In the Se- rats, erythrocyte glutathione peroxidase activity and plasma selenium concentration were strongly reduced as compared to pair-fed selenium-adequate rats (Se+). Weight and tail length were reduced by 31% and 13% in the Se- rats, respectively ( $p < 0.001$ ). The Se- diet was associated with a 68% reduction of pituitary growth hormone (GH;  $p = 0.01$ ) and a 50% reduction of plasma insulin-like growth factor I (IGF-I;  $p < 0.001$ ). Plasma calcium was lower and urinary calcium concentration was greater in Se- rats. This group had a 2-fold increase in parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D<sub>3</sub> [1,25(OH)<sub>2</sub>D<sub>3</sub>] in plasma. Plasma osteocalcin and urinary deoxypyridoline were reduced by 25% and 57% in the Se- rats ( $p < 0.001$ ). Selenium deficiency resulted in a 23% and 21% reduction in bone mineral density (BMD) of the femur and tibia ( $p < 0.001$ ) and this effect persisted after adjustment for weight in a linear regression model. A 43% reduction in trabecular bone volume of the femoral metaphysis ( $p < 0.001$ ) was found in Se- rats

**Other Minerals.** Data indicate that adding 25 mg/kg of dietary **nickel** to a poultry diet will have a positive influence on bone strength characteristics and performance (Wilson *et al.*, 2001). **Aluminum** in feed (0.3%) reduces tibiotarsal length (Johnson *et al.*, 1992), but levels as low as 0.1% reduced bone strength and concentrations of Ca, P, Mg and Zn. Supplementation of **boron** (5 -25 mg/kg of feed) to diets with inadequate (0.25 MIU) or adequate (2.0 MIU) vitamin D<sub>3</sub> content increases significantly the tibia calcium content and reduces its zinc content, resulting in stronger bones (Kurtoğlu *et al.*, 2005).

Deficiency of **copper**- Cu (< 1 ppm) was shown to decrease collagen crosslink formation and to lower mineralization (Osphal *et al.*, 1982). Banks *et al.* (2004) observed that even though the body weight gain was not different ( $P > 0.05$ ) among groups fed with Cu lysine (Cu-Lys), Cu sulfate (Cu-Sul), Cu citrate (Cu-Cit) and Cu chloride (Cu-Cl), the supplementation with 250 ppm of Cu from Cu-Lys resulted in chicks having greater toe and tibia ash weights and percentage as compared with birds supplemented with Cu-Sul. They also observed that supplementation with 250 ppm Cu from Cu-Cit or Cu-Sul resulted in decreased apparent P retention. Supplementation with 250 ppm Cu-Cl or Cu-lys, however, improved apparent P retentions as compared with Cu-Cit or Cu-Sul.

**Fluoride** in the form of sodium fluoride (~100 ppm) increases bone density and breaking strength in chickens (Merkely and Miller, 1983; Lundy *et al.*, 1992). Fluoride seems to increase the activities of osteoblasts and accelerate mineralization, while inhibit the activities of osteoclast (Dolegowska *et al.* 2003). Under normal dietary conditions fluoride levels do not cause risks related with bone fractures (Holick et al, 2004) as it has been previously suggested (Leeson *et al.*, 1995).

**Magnesium** (Mg) at dietary levels of 0.3% causes shortening, twisting and bowing of the tibiotarsus with a concomitant reduction in bone ash (Lee *et al.*, 1980). Microscopic evaluation showed rachitic-type lesions, with a widened and lengthened growth plate, excessive osteoid seams on endochondral bone and osteoid or capped metaphyseal blood vessels with few associated osteoblasts. It is important to limit the Mg content in the diets,

since commercial broiler diets using dolomitic limestone (20% Mg) can contain up to 0.4 – 0.5% of Mg.

**Cadmium** (Cd) is a heavy metal that is widely distributed in the environment and is considered the most toxic of the heavy metals with a maximum tolerable level of 0.5 ppm (NRC, 1980; 1994). Low-level long-term exposure to Cadmium (Cd) causes skeletal damage, suppressed humoral and cellular immunity, and thyroid dysfunction. In recent years, Cd poses a potential environmental hazard due to increases in its industrial use. Chickens are exposed to cadmium by feed and ground water contaminated by leaching (Rambeck and Guillot, 1990; 1996; Vodela *et al.*, 1997a, b; Linden *et al.*, 1999). Other potential sources include mining and smelting operations, corrosion of metal-plated iron, discarded cadmium-chloride products, and the use of urban sewage sludges to fertilize pastures or croplands.

The most likely source of contamination in the animal feed industry is in conjunction with the use of zinc sulfate or poorly processed zinc ores as sources of supplemental zinc. Addition of 60 ppm zinc to the diet would contribute only 0.1 ppm cadmium from the zinc oxide containing 1,290 ppm of the element and about 0.008 ppm from the oxide containing 79 ppm. Sources of zinc sulfate generally contain greater amounts of cadmium than oxide forms and should be analyzed carefully prior to use (NRC, 1980).

Relatively large quantities of Cd are found in commercial phosphate fertilizer and phosphate sources (0.1 - 67 ppm with an average of  $9.4 \pm 14$  ppm). Thus, the increases in soil and plant Cd contents may lead to increases in dietary Cd for poultry. For example, some soybeans can contain between 55.7 and 73.5 ng Cd/g (Zhang *et al.*, 1998). Addition of the highest cadmium-containing phosphate to poultry diets at 2% would add approximately 1.5 ppm cadmium while the average concentration (9.4 ppm) would add less than 0.2 ppm of the element. The defluorinated phosphates are practically devoid of cadmium, probably as a result of volatilization of element during thermal processing. In fact, the cadmium content of practical-type diets for poultry generally is about 0.05 to 0.35 ppm (NRC, 1980).

Gupta and Kar (1999) showed that the administration of cadmium chloride (2.5 mg/kg body weight/day) to chickens daily for 15 days decreased serum triiodothyronine (T<sub>3</sub>) concentration (by 68.75%) without altering the levels of serum thyroxine (T<sub>4</sub>). Hepatic 5'-monodeiodinase (5'D-I) and superoxide dismutase (SOD) activities were also decreased (by 90.47% and 20.81% respectively) with a concomitant increase in lipid peroxidation (LPO, by 206.25%). The administration of the antioxidant vitamin E at 5 mg/kg body weight on alternate days, to cadmium intoxicated chickens or rats restored thyroid function by maintaining normal hepatic 5'D-I activity and serum thyroid hormone concentrations (Gupta and Kar, 1999). Vitamin C administration restored serum T<sub>3</sub>, but no T<sub>4</sub> levels (Gupta and Kar, 1998). Both vitamins prevented cadmium-induced increase in LPO.

Mineral content of the liver is modified according to Cd level: iron, magnesium and selenium decreased while copper, zinc and manganese increased with increasing Cd levels. Iron is the most strikingly affected metal, falling to one-fifth of control values at high dietary Cd exposure. In this dosage group, selenium decreased to 36% of mean control concentrations while zinc increased to 168%. This mineral imbalance, especially depleted iron stores, can contribute, at least in part, to the Cd-associated risk of osteoporosis (Noel *et al.*, 2004). Cadmium also has an antagonistic activity in Ca metabolism and reduction in serum P

concentrations due to either increased P excretion because of tubular damage in kidney or suppressed absorption of this element or both (Uyanik *et al.*, 2001).

**Protein and specific amino acids.** Broilers and turkeys fed low crude protein diets normally show fewer leg defects (Leeson *et al.*, 1995). However, this effect might be caused by reduction of growth rate (Ferket and Sell, 1989; Waldroup *et al.*, 1998) and performance that is not recovered even after re-feeding higher amino acid levels.

Skinner *et al.* (1991) observed that when the total amino acid levels were increased by 20% above the requirements of the birds, a significant reduction in tibia mineralization, weight and length occurred among birds consuming diets containing 0,5% and 1,0% calcium. When the birds consumed 1.0% calcium in the diet, tibia weight increased with decreasing amino acid levels, however, when the diet contained 0.50% calcium no increase in tibia weight occurred with elevation in amino acid levels. Then, marginal levels of calcium in diets with higher levels of amino acids can affect bone development.

In a study of bone development of broiler chickens fed different amino acid and calcium levels with respective recommendations of 80, 100 and 120% and 50, 100 and 150% of NRC guidelines (1984), Sekine *et al.* (1994) noted an interaction between the factors evaluated and bone density. Diets containing 150% of recommended calcium levels did not promote a greater resistance of the tibia to fracture as compared to rations containing 120% amino acids. In addition, calcium levels affected the longitudinal growth of the tibia and promoted bone calcification. On the one hand, amino acid levels acted on transverse growth and facilitated the formation of bone matrix as well as calcification. On the other hand, several authors have failed to observed significant interactions between amino acids and minerals (Soares da Silva *et al.*, 2003). Soares da Silva *et al.* (2003) observed that regardless of the level of amino acids, the diets with low calcium level reduced bone density, tibia weight and spongy layer thickness for the Cobb strains but not for the Avian Farms genetic line demonstrating that some strains are more sensitive to a reduction in dietary calcium. Driver *et al.* (2005) concluded that current recommended level of 1.0% for broiler starter diets is adequate, and 0.9% is even excessive for grower diets independently of the level of protein in the diet.

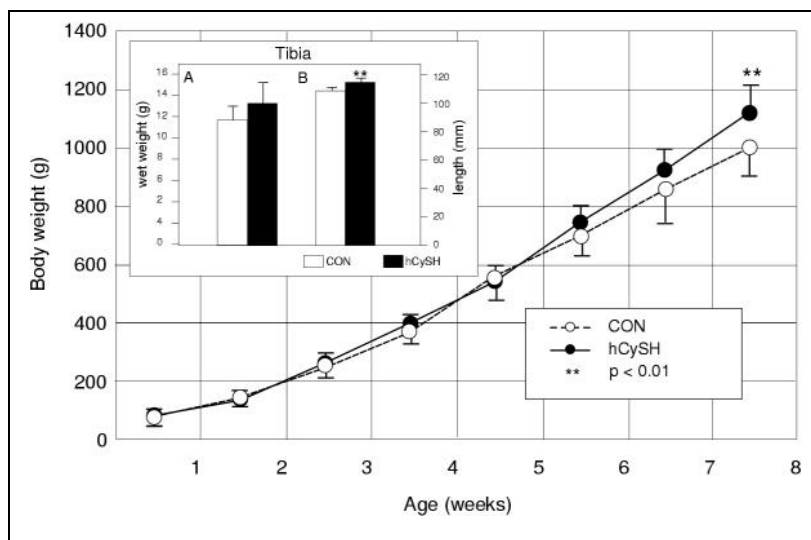
Marginal deficiencies of amino acids such as valine can occur when low crude protein diets are utilized. Farran and Thomas (1992) showed that birds fed a starter valine deficient diet have increased incidence of leg problems, and related this to reduced hydroxyproline availability and also to increased calcium excretion in the urine.

**Sulfur amino acid metabolism and leg problems.** Sulfur amino acids seem to play the main lead role on the effect of excess of protein in bone development. Diets with excess of sulfur amino acids can interfere with folic acid, pyridoxine, and vitamin A metabolism and increase their requirements. All these vitamins have been related to bone development and treatments for leg problems in avian species.

Diets with an excess of methionine and marginal deficiencies of folic acid and pyridoxine (Vitamin B<sub>6</sub>) cause hyperhomocysteinemia. Marginal deficiencies in vitamin B<sub>6</sub> is common in the poultry meat industry because it is commonly formulated under the NRC (1994) requirement (Tables 2 and 3) to promote better feed conversion. A recent study has shown that homocysteine could inhibit the conversion of retinal to retinoic acid in avian embryos (Limpach *et al.* 2000). In the same way, the requirements of vitamin B<sub>6</sub> for maximal growth

was found to increase ( $P < 0.01$ ) from 0.73 to 1.05 mg/kg, a 44% increase, when 10 g/kg excess methionine was present in the diet. Chicks were fed seven graded doses of supplemental pyridoxine (PN) in diets that contained either adequate (2 g/kg) or excess (12 g/kg) methionine. Indeed, this level of excess dietary methionine depressed ( $P < 0.01$ ) growth at all PN dose levels  $<1$  mg/kg, but not at PN doses of 1.2 or 1.4 mg/kg (Scherer and Baker, 2000).

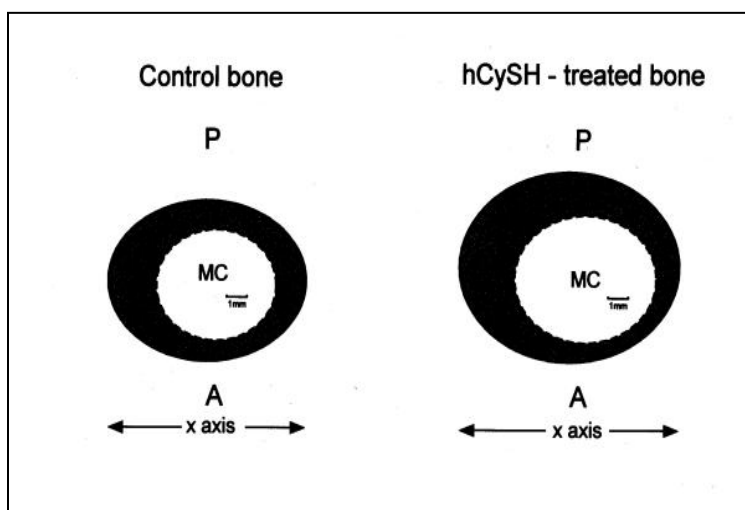
The hyperhomocysteinemic chickens have higher plasma levels of hCySH, methionine, cystathionine, and inorganic sulfate, but calcium, phosphate, and other indices of osteoblast metabolism are not different from controls (Massé *et al.* 1995, 1996). These hyperhomocysteinemic chickens grew faster, had heavier and longer tibias (Figure 1), and had accelerated radial and longitudinal bone growth with more advanced ossification of epiphyses and greater architectural efficiency of the diaphyseal cross-sectional design. Massé *et al.* (2003) observed that radiographs of the lower limbs showed generalized osteopenia with distinct metaphyseal and suprametaphyseal lucencies.



**Figure 1.** Growth curves (mean  $\pm$  SD of body weights) for control (CON) and hyperhomocysteinemic (hCySH) groups of broilers. hCySH-fed chickens were significantly heavier at the end of the 8-week experiment (\*\* $P < 0.01$ ). Inset: (A) tibial wet weight in grams; (B) length of tibia in millimeters (\*\* $P < 0.01$ ). Source: Massé *et al.*, 2003.

Hyperhomocysteinemia causes an abnormal bone collagen matrix defect, as chondrodysplastic cartilage changes in tibial cross-sectional contour (Figure 2), and the previously described radiological changes that indicate points of bone weakness. Although biomechanical testing of the tibiae, including maximal load to failure and bone stiffness, indicate stronger bone, strength was proportional to the increased length and cortical thickness in the hCySH-supplemented group. Bone ash weights and infra red-spectroscopy of cortical bone showed no difference in mineral content or mineral crystallization, but there were chemical abnormalities of bone mineral composition such as higher  $\text{Ca}^{2+}/\text{PO}_4^{3-}$  and lower  $\text{Ca}^{2+}/\text{CO}_3^{2-}$  molar ratios than in controls.

These findings may have applications in the meat poultry industry where diets are formulated with high levels of methionine to maximize breast meat yield responses and guarantee good feathering, however some of the bone fractures and fragility could be due to excess of methionine and marginal deficiency of pyridoxine (Tables 2 and 3).



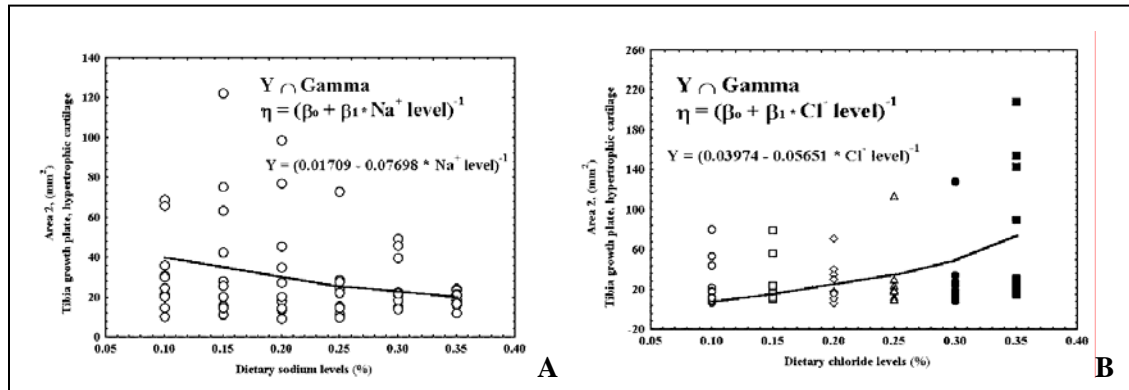
**Figure 2.** Mean mid-shaft tibial cross-sectional contours for control and homocysteine (hCySH)-treated chickens. hCySH-treated cortical bone is asymmetric with an eccentric medullary cavity. Its greater thickness on one side of the X axis was also noticeable on radiographs. A: anterior cortex of tensile side; P: posterior cortex of compressive side; MC: medullary cavity; — periosteal cortical bone; - - - endosteal cortical bone. Bar = 1 mm. **Source:** Massé *et al.*, 2003.

**Feeding and Management Practices.** Quantitative feed restriction and microelement supplementation at 7 days of age reduced mortality from ascites and leg problems and permitted compensatory growth sufficient to equal the production characteristics of the control group at 49 d of age (Camacho *et al.*, 2004). Feeding whole grain can decrease mortality and leg problems in turkeys (Bennett *et al.*, 2002), or no have clear effects on leg problems in broilers (Lippens *et al.*, 2000). Bruno *et al.* (2000) observed that feed restriction and high environmental rearing temperature reduce long bone growth. In their study bone breaking strength was not affected by bird age, feed restriction nor rearing ambient temperature, but the calculated BW/BL index was reduced by heat exposure. High environmental rearing temperature reduced bone length and width at 42 d of age. The common management practice of adding organic acids such as citric acid enhances dialysability of calcium, magnesium, manganese and zinc, but at the same time increases lead and cadmium dialysability (Walter *et al.*, 1998).

**Electrolyte balance.** The dietary electrolyte balance (DEB) value measured by the effect of  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Cl}^-$  concentration (mEq/Kg) affects the blood acid-base balance status of animals. Blood pH, partial pressure of  $\text{CO}_2$ , bicarbonate ( $\text{HCO}_3^-$ ),  $\text{CO}_2$  tension ( $\text{TCO}_2$ ), and base excess are significantly affected by small dietary modifications of  $\text{Na}^+$  or  $\text{Cl}^-$  (Oviedo-Rondón *et al.*, 2001; Murakami *et al.*, 2001). It is known that bone mineralization process are highly pH dependent (Carano *et al.*, 1993; Farquharson *et al.*, 2003, Bushinsky, 2004; Pines *et al.*, 2005).

Numerous poultry researchers have reported higher leg problem incidence in cases of metabolic acidosis in broilers and turkeys (Leeson *et al.*, 1995). For example, Halley *et al.* (1987) observed that chicks having the lowest base excess had higher incidences of both leg disorders. Whitehead (1989) cites evidence that metabolic acidosis, caused by high chloride levels in the feed, can reduce the production of  $1,25(\text{OH})_2\text{D}_3$  in the kidney and higher production of 24-25 DHCC. Additionally, chronic metabolic acidosis depletes proton buffers in bones, with phosphate exceeding that of  $\text{HCO}_3^-$  at the expense of the bone mineral (Bushinsky *et al.*, 2003). Oviedo-Rondón *et al.* (2001) and Murakami *et al.* (2001; 2003) have consistently observed in several experiments that incidence and severity of TD,

measured by the increment in the hypertrophic area of the the tibia growth plate, increases by  $\text{Cl}^-$  and is reduced by  $\text{Na}^+$  (Figure 3). We recommend that dietary DEB should be maintained around 250 mEq/kg for maximum growth and reduce TD incidence.



**Figure 3.** A. Nonlinear model (gamma distribution and link function inverse power) describing areas of hypertrophic region of tibial growing cartilage of broiler chickens in relation to dietary sodium levels at 22 d of age (A) or chloride levels during growing phase at 42 d of age. (A) Supplemental sodium (0.05 to 0.25%) was provided from sodium bicarbonate (0.19 to 0.93%). (B). The supplemental chloride (0.05 to 0.30%) was provided by sodium chloride (0 to 0.34%). **Source:** (A) Oviedo-Rondón *et al.*, 2001 and (B). Murakami *et al.*, 2001.

**Fatty acids.** Dietary lipids alter the fatty acid composition of bone polar and neutral lipids (Taylor, 1998), and the concentrations of prostaglandin and IGF-I in bone and consequently affect longitudinal bone growth (Watkins *et al.*, 2000, Watkins, 2002). Acidic phospholipids in matrix vesicles are an integral part of  $\text{Ca}^{2+}$  and Pi complexes initiating mineralization in epiphyseal cartilage. Growth cartilage chondrocytes of broiler chickens selectively accumulate fatty acids from the diet (Xu *et al.*, 1994a). Although Omega-3 polyunsaturated fatty acids are readily taken up by chondrocytes, the essential fatty acid linolate (omega-6) do not concentrate in these cells. This indicates that growth cartilage in young chickens may be sensitive to excessive amounts of certain unsaturated fats. Moderate amounts of saturated fats (Watkins *et al.*, 1993) and supplemental vitamin E (Xu *et al.*, 1994b) enhanced bone formation. These effects are due to more available arachidonic acid for PGE2 production, and altered concentrations of IGF-I, in plasma, cortical bone and epiphyseal cartilage.

Taylor (1998) observed that the lipid composition of the maternal diets stimulates growth rate, bone development and strength of the progeny. The tibiae diameter of chickens hatched from eggs collected from breeders fed diets containing poultry fat (3%) were significantly larger than the ones from breeders fed menhaden oil at 14 and 28 days of age. Chickens coming from breeders fed diets containing soybean oil had tibias with higher values for shear force and breaking strength than the ones coming from hens fed poultry fat and menhaden oil.

**Mycotoxins, production and immunological stresses.** Aflatoxins and ochratoxins cause bone fragility and affect vitamin D metabolism (Huff *et al.*, 1980; Duff *et al.*, 1987). While feed contamination with fumonisin B<sub>1</sub> (Wu *et al.*, 1995) and fusaric acid (Chu *et al.*, 1993) do not have good correlation with TD or any other leg disorders.

Environmental stress (Rath *et al.*, 2000) and immune response to bacterial challenge simulated by *E. coli* lipopolysaccharide injections (Mireles *et al.*, 2005) reduce tibia bone calcium content and breaking strength in chickens. The same responses could be expected in turkeys. The severity of this effect is mediated by glucocorticoids and cytokines, and is age dependent.

## Conclusions

Leg abnormalities in meat poultry is a very complex problem that can be influenced by many different factors. The genetic modifications of fast-growing broilers and turkeys to their hormonal balance (thyroid, GH and IGF) on systemic and tissue-specific functions, and the number of cellular vitamin D receptors in bone tissues could play important roles in the predisposition to different types of bone developmental disorders. Breeder nutrition, incubation conditions, management (brooding) practices, and the physiological responses to environmental stress and immunological responses might affect the field incidence of these developmental problems. Chicks and poults with high alterations in their bone regulatory mechanisms might not respond to any dietary manipulation directed to solve leg problems. Thus, genetic selection comes again as the less expensive and main strategy to solve these issues in the long term.

To understand a specific skeletal problem observed in the field and develop a plan to solve it, we must study several parameters. Although bone mineralization and density are good markers of bone development, it is very important to evaluate the biochemical composition of bone collagen and the biomechanical properties of the bone by testing tensile, torsion and fatigue. Along with good feed quality and sufficient dietary nutrient levels typically used in the poultry industry, attention should be placed to levels of highly bioavailable sources of zinc, selenium, copper, pyridoxine, biotin, and sources of vitamin D. Moreover, appropriate levels of Ca, P, Na, Cl and good balance between  $\Omega 3$ :  $\Omega 6$  fatty acids according to the stage of development are important. It is noteworthy to assess the effects of high methionine-low pyridoxine diets on bone development and resistance to fractures in broilers and turkeys under commercial conditions. Additionally, strategies to avoid enteritis, fat and oil rancidity, mycotoxins, and cadmium contamination should be placed at the feed mill and at the farms to reduce skeletal disorders in poultry.

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# Overview of the Immune Dynamics of the Digestive System

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## Summary

Activation of the immune system of poultry can divert nutrients away from growth. As the digestive tract is a major site of pathogen exposure, an understanding of the function and regulation of the immune system may help nutritionists improve performance, and minimize the potential negative impacts of the reduction or loss of the use of growth-promoting antibiotics in poultry production. The role and structure of the avian immune system is similar to that of mammals, although there are several mechanistic and regulatory differences. The innate immune system responds non-specifically to foreign molecules, whereas the acquired immune response involves recognition of specific antigens unique to individual pathogens.

## INTRODUCTION

The wild progenitors of modern chickens and turkeys inhabited an environment very different than commercial production systems. Modern birds live in a much more controlled environment, where disease challenges are much different. As well, a successful resolution of an immune challenge for a wild bird would be survival, whereas success under commercial terms is defined not only as survival, but in terms of continued rapid, efficient growth or egg production.

## ROLE OF THE GUT

The primary function of the digestive tract is the assimilation of nutrients. However, because the material ingested by birds contains not only nutrients, but inert material, anti-nutritional factors, and potential pathogens, another important function of the gut is exclusion.

## OVERVIEW OF IMMUNE RESPONSES IN POULTRY

The function of any immune system is the differentiation of “self” and “non-self”, and the appropriate response to identification of each. Birds have both non-specific and specific immune mechanisms to respond to potential infectious threats from bacteria, viruses, parasites and other antigenic material. The two aspects of the immune response are innate (non-specific) and acquired (specific) immunity. These two types of responses are usually coordinated, with pathogenic challenges being initially processed by the innate system, and if necessary, the subsequent activation of the acquired response. The general mechanisms of immunity are identical in chickens, mammals and most other vertebrates (Erf, 2004; Jeurissen et al., 1994).

Both innate and adaptive immunity are required for host survival and health. The innate response is a generalized response to broad categories of foreign (non-self) immunological challenges, such as pathogens. The innate response is systemic, and affects host physiology. Activation of this system can divert considerable amounts of

nutrients away from growth (Barnes et al., 2002). Acquired immunity targets specific antigens, and response to a particular pathogen involves a very limited subset of lymphocytes. The acquired response is exquisitely specific and targeted; activation of an acquired immune response requires very little in the way of nutrients, and causes very little change in metabolism of the bird (Humphrey and Klasing, 2004; Klasing and Calvert, 1999). For the host to be fully protected, the two aspects of the immune system must work together. Despite the potential negative effect of an innate immune response on growth and productivity of poultry, attempts to improve performance through manipulation of innate immune function must be done with this in mind. It may well be that the key to reducing the impact of immune system activation on bird performance is to reduce exposure to foreign antigen.

### ***Innate immunity***

Innate immunity involves the non-specific mechanisms the bird possessed to resist disease. The innate immune system is functional at hatch, and development continues to occur during the first week of life (Wells et al., 1998). This is the first line of defence against invading pathogens. The skin and mucosal surfaces are the first aspect of innate immunity, acting to exclude pathogens by barriers to entry (Lillehoj and Chung, 1992). Any foreign organism or antigen crossing these barriers is encountered by intraepithelial leukocytes, including macrophages, dendritic cells, heterophils, natural killer cells and T lymphocytes (Jeurissen and Janse, 1996; Lillehoj and Chung, 1992; Banchereau and Steinman, 1998). All but the lymphocytes are generally considered to be cells of innate immunity. Innate immune cells have several functions, including the recognition and control of invading pathogens as a first line of immunological response, as well as antigen presentation and subsequent activation of the mechanisms of acquired immunity.

The innate response is non-specific, in that the immune cells do not recognize specific antigen, but generalized, conserved molecules common across many pathogens through pathogen-associated molecular patterns (PAMP; Humphrey and Klasing, 2004) and other receptor-mediated methods (Qureshi, 2003). This non-specific recognition of a wide array of foreign invaders allows the innate immune system to respond much more rapidly to challenges than the acquired immune response (Merlino and Marsh, 2002). The innate immune response is essential immediately following pathogen exposure during which the acquired immune gradually response takes on a greater role in host defence over days or weeks.

### ***Acquired immunity***

Acquired (adaptive) immunity must be developed by the bird in response to a unique, novel pathogen. The bird must first activate the mechanisms responsible for dealing with that particular invader. Acquired immunity is specific, heterogeneous and has memory, meaning a specific pathogen will be recognized by the immune system in a particular way, different pathogens elicit different responses, and re-exposure to the pathogen will often result in a more rapid, effective response than was elicited following the first exposure. Acquired immunity involves cell mediated and humoral aspects (Qureshi et al., 1998). In cell-mediated immunity, cells infected with a foreign pathogen are destroyed via interaction between the infected cell and an effector cell such as an activated T cell (Qureshi et al., 1998). Humoral immunity uses antibodies produced by B cells in response to an antigenic challenge. The two types of lymphocytes are different

phenotypically; T cells express T cell receptor complex on their surface whereas B cells express immunoglobulin on their surface (Gobel, 1996). The regulation and effectiveness of avian adaptive immunity is comparable to that in mammals (Erf, 2004).

### ***Cell-mediated immunity***

The cell-mediated aspect of the acquired immune system is directed by T lymphocytes (T cells). Cytotoxic T lymphocytes (CTL) recognize infected cells, and release mediators (lymphotoxins) that cause programmed cell death. T helper lymphocytes (TH cells) direct the immune response, secreting lymphokines that stimulate CTL and B cells to grow and divide, attract heterophils, and potentiate engulfment and destruction of microbes by macrophages. Suppressor T cells prevent excess damage to the host's own tissues by inhibiting the activity and production of cytotoxic T cells following successful elimination of the pathogen. Memory T cells recognize and respond rapidly when a particular pathogen is recognized subsequently (Erf, 2004).

### ***Humoral Immunity***

Humoral immunity, mediated by antibodies carried in the blood, is the second type of adaptive response is, the response. B lymphocytes are the effector cells of the humoral response. When a naïve B cell encounters its specific antigen in the blood, a surface immunoglobulin acts as a receptor to allow the B cell and antigen to bind (Janeway et al., 2001). The B cell must then bind to an antigen-specific, activated TH2 cell in order to become activated. The TH2 cell releases cytokines, which in turn primes the B cell to undergo an asexual mitotic reproduction of the particular cell (clonal selection). Most of the clones become plasma cells, which after an initial lag are capable of producing immense numbers of antibody molecules.

As with naïve T lymphocytes, there are very few B cells that will recognize and react with a particular pathogen. The vast range of specificities in B cell antigen recognition are the result of immunoglobulin gene rearrangement in pro-B cells (Ratcliffe, 2002).

When the pathogen has been eliminated and the antigen is no longer present in the body, the antigen-specific B cells begin to die off. Some of the B cells, however, become long-lived memory cells, present at 10-100 fold higher levels than initially, but also more potent in antibody-secreting capacity than unprimed B cells specific for the same antigen (Janeway et al., 2001). Unlike most mammals, this B cell development early in the bird's life takes place in the bursa of Fabricius (Sayegh et al., 2000), rather than the bone marrow (Ratcliffe, 2002).

## **PROGRESSION OF THE IMMUNE RESPONSE**

When a pathogen is encountered for the first time, it must first be recognized by the bird as "non-self". During the recognition phase, innate immune cells engulf and kill the pathogenic invader and process the antigen. During the activation phase, recruitment, proliferation and activation of lymphocytes takes place, and the effector phase results in the elimination of the pathogen using antigen-specific mechanisms. The extent of involvement of the innate, cell-mediated and humoral aspects of the immune system will vary depending on the type of pathogen, the severity of the challenge, the overall health and nutritional status of the bird, and many other factors. The innate immune response is

often sufficient to resolve the challenge. In that case, the acquired response is not activated, and there will be no immunological memory of the pathogen.

Once the pathogen has been cleared from the bird, the antigen is no longer present to stimulate the antigen-specific T and B lymphocytes. The clones begin to die off, although a few memory cells remain in circulation. The memory cells allow a more rapid response the next time that the antigen is encountered.

If the innate immune system has previously been successful in resolving the challenge posed by a particular pathogen, no immunological memory will exist, and the second exposure is handled in the same manner as exposure to a novel pathogen. If, the acquired immune system has previously responded to the pathogen, memory T and B cells may remain. Memory of particular pathogens may decrease with time, at a rate that varies with pathogen type, severity of the challenge, and other factors. As long as memory cells remain, the next time the pathogen is encountered, a much more rapid response by the acquired immune system can be produced. The innate immune system is still required for antigen processing and presentation, as well as the initial defence of the bird. However, the transition from innate to acquired immune system dominance will be much more rapid.

## **GASTROINTESTINAL TRACT IMMUNITY**

The mucosal surfaces of the gut is one of the main routes of access of pathogens to the bird, therefore the gut has an essential role in protection from disease (Lillehoj and Trout, 1996; Bar-Shira and Friedman, 2005, Schat and Myers, 1991; Muir et al., 2000). An appreciation for the role of the GI tract, the distribution and function of the immune system within the gut, and means of manipulating those interactions to the benefit of birds and producers is essential for continued safe, efficient production of poultry products for human consumption.

### ***Epithelial and Mucosal Barrier***

The epithelial surface of the bird is an essential component of immunity, preventing access to the host by pathogens (Jeurissen and Janse, 1996). The mucosal surface, which includes the GIT, is the largest surface of interaction between the bird and the outside world. The intestinal tract is exposed to a wide variety of foreign molecules and microbes. The mucosal barrier and gut-associated lymphoid tissue (GALT) are essential for protection from invasion by pathogenic organisms. Pathogenic organisms are trapped in the mucosa, where they are inactivated by secreted products such as secretory IgA (Lillehoj and Trout, 1996; Muir et al., 2000), lysozyme, and other antimicrobial compounds (Muller et al., 2005). The inactivated microbes are unable to colonize and proliferate, and are passed out of the digestive tract.

### ***Gut-Associated Lymphoid Tissue***

The GALT is one of the largest secondary immunological organ in the body. GALT is comprised of immune tissues and cells found within the tissues of the digestive tract. Cells involved in antigen presentation, immunoregulation, and effector function are present in GALT (Lillehoj and Trout, 1996). The immune cells can occur as scattered, individual cells responsible for surveillance of foreign material that may enter the bird's tissues, or as discrete lymphoid aggregates (Jeurissen et al., 1994; Bar-Shira et al., 2003)

where antigen presentation to the appropriate effector cell is more likely due to the concentrated populations of immune cells. GALT lymphoid aggregates in birds include the esophageal tonsil, Peyer's patches, cecal tonsils, the bursa of Fabricius, and localized lymphoid follicles that form near a site of infection (Jeurissen and Janse, 1996; Lillehoj and Trout, 1996; Muir et al., 2000; Bar-Shira et al., 2003). Most immune cell types are present in the lamina propria of the gut, including macrophages, granulocytes, plasma cells, effector T lymphocytes and memory lymphocytes (Bar-Shira et al., 2003). The distal portion of the GIT has a greater GALT presence than more proximal regions because it is exposed to higher microbial loads than other portions of the digestive tract (Bar-Shira et al., 2005).

## **CHALLENGES AND OPPORTUNITIES**

### ***Antibiotic Growth Promoters***

Growth promoting antibiotics have been used to great advantage in the poultry industry since the 1950's (Dibner and Richards, 2005). The mode of action is not entirely understood, and different antibiotics have different mechanisms. One of the primary effects of sub-therapeutic levels of antibiotics seems to be a reduced the activation of the inflammatory response (Solomons et al., 1993; Apajalahti et al., 2004). Consequently, nutrients are available for growth rather than for the systemic metabolic changes associated with inflammation (Humphrey and Klasing, 2004; Roura et al., 1992).

In many areas of the world, growth-promoting antibiotics have been removed partially or entirely from feed, either through legislative action or through companies voluntarily eliminating antibiotic growth promoters to address consumer concerns (Dibner and Richards, 2005; Bedford, 2000; Dibner and Richards, 2004). It is reasonable to assume that North American and many European countries will drastically change the use of antibiotic growth promoters in the near future. There have been intensified efforts to find new ways of reducing the exposure of pathogenic microbes to the bird, or modulating the way in which the bird responds to such challenges. In the following section, several potential approaches to minimize the impact of the loss of growth promotants are discussed.

### ***Genetic selection for disease resistance***

Genetic selection for altered immune response has been used effectively by the poultry industry in the past. Perhaps the most notable application has been the selection for certain MHC haplotypes conferring resistance to Marek's disease in chickens (Lamont, 1998; Zekarias et al., 2002). Selection of poultry for traits in addition to growth and efficiency will likely continue play a major role in the advancement of health and productivity in poultry (Fulton, 2004). It should be noted that in general, activation of the immune response has a negative effect on growth rate, and selection of lines of chickens for increased specific responses result in lines of birds with poorer growth rate (Qureshi and Havenstein, 1994; Martin et al., 1990; Parmentier et al., 1996). Genetic improvements in the ability of birds to resist various diseases may therefore come at a cost of reduced growth rate, or slowed advances in increases in growth rate. Different pathogens elicit different types of immune responses, and selection for resistance to one particular disease may make the bird more susceptible to other diseases. A greater

understanding of immune system regulation is necessary for application to poultry breeding programs in the future.

### ***Exogenous Enzymes***

Wheat or barley are used in poultry diets in many parts of the world. Although more readily available than corn in many locations, these cereals may contain high levels of indigestible, soluble non-starch polysaccharides (NSP). High NSP diets cause increased water intake and litter moisture, increase digesta viscosity which decreases in mixing (thus reducing enzyme-substrate interactions and absorption of released nutrients), and decreased production (Choct and Annison, 1990; 1992a; 1992b; Choct et al., 1995; 1996; 1999). NSP alter intestinal microbial populations of animals (Apajalahti et al., 2004; Hogberg et al., 2004), making this an exciting area of research. High-viscosity, wheat-based diets fed to poultry diets may favour pathogenic bacteria such as *E. coli*, salmonella, clostridia and campylobacter (Williams et al., 2003; Bjerrum et al., 2005; Murphy et al., 2004; Engberg et al., 2004), possibly due to the creation of a more anaerobic environment. Decrease mixing in the gut appears to be associated with the increased digesta viscosity reduces enzyme-substrate interactions, leaving more feed undigested in the GIT. Substrate liberated following enzymatic cleavage will be less likely to migrate to the absorptive surface of the intestine, making it more available for use by intestinal microbes, including pathogens. Recently, it has been shown that, similar to the situation with wheat, corn source and quality can affect microbial populations in poultry; these effects can be modulated with exogenous dietary enzymes (Korver et al., unpublished data).

### ***Probiotics and Prebiotics***

Probiotics are cultures of live bacteria that are fed to poultry to hasten the development of a stable, “normal” gut microflora. This usually involves feeding the cultures to poultry beginning early in the life of the bird to rapidly establish a protective layer of beneficial bacterial lining the GI tract. Because the chick hatches with a sterile digestive tract, microbial colonization begins almost immediately following dosing. The number and type of microbial species present in the GI tract change as the microflora develops. Some species appear and disappear (Guan et al., 2003); all the while the population becomes more complex in its makeup. During the early part of a bird’s life, microbial populations are in a state of flux (Lu et al., 2003). The sooner a healthy, stable gut microflora occupies all of the ecological niches in the gut, the less likely colonization by pathogens becomes. For pathogenic bacteria to proliferate in the gut, they must have attachment sites. Without attachment sites, the microbes are swept along with the digesta and removed from the bird via defecation.

The microbial community of the intestinal tract is exceedingly complex, and the most complex of probiotic preparations seem to be most protective for the bird (Waters et al., 2005). Undefined probiotic cultures are not approved in many countries on the basis that, without knowing exactly which microbial species are present in the preparation, pathogens may inadvertently be fed to the birds, thereby increasing, rather than reducing colonization by pathogens. Because of the complexity of creating and maintaining complex defined culture probiotics, most commercially available preparations contain one, or at most a few, species of bacteria, generally Lactobaccilli. These defined, simple

preparations are usually far less effective in conferring protection against pathogen colonization than the more complex defined or undefined cultures (Waters et al., 2005).

In order for bacteria to thrive in an ecological niche, environmental variables must be suitable (eg. temperature, pH, presence or absence of O<sub>2</sub>, etc.), and the bacteria must have access to sufficient quantity and quality of nutrients to allow it to thrive. Prebiotics are molecules included in the diet that are intended to “feed” the beneficial microflora of the gut (Guo et al., 2004). When probiotics and prebiotics are provided together in the diet, not only the introduction, but also the maintenance of beneficial bacterial populations may be accomplished (Le Leu et al., 2005; Macfarlane et al., 2005).

### ***Nutritional Immunomodulators***

The cells of the immune system require nourishment to function properly. Many dietary components appear to have effects on the immune system beyond simply providing nutrients. Although the list of such nutrients is extensive (Kidd, 2004; Klasing, 1988; Klasing, 1998), an example is given below.

### ***Omega-3 Polyunsaturated Fatty Acids***

Omega-3 polyunsaturated fatty acids (n-3 PUFA) have been used in poultry rations to alter the inflammatory response and reduce the systemic effects of pathogenic challenges. Relatively low amounts of fish oil (0.5-2%) were found to have reduced reduced IL-1 production by avian macrophages, and improved broiler growth rate when the birds were challenged with bacterial lipopolysaccharide (Korver and Klasing, 1997). Similar levels of fish oil resulted in increased inflammatory cell infiltration to the site of an experimental coccidiosis challenge, but a decrease in the systemic effects of infection (Korver et al., 1997). This suggests that dietary fish oil may be a means of somewhat uncoupling the localized immune response necessary for dealing with a pathogen from the systemic, production-suppressing effects of inflammation.

### ***Exogenous dietary antibodies***

For GIT pathogens to infect a bird, they must be able to get through the protective barrier of the mucosal surface. This can happen when the gut lining is damaged, such as in the case of secondary necrotic enteritis following tissue damage following coccidiosis (Van Immerseel et al., 2004), or through active invasion by the pathogen. Exogenous antibodies can be fed to poultry in order to bind up antigens present on the surface of pathogens, causing the microbes to clump together and prevent colonization of the GI tract. The antibodies are relatively large molecules, and are not absorbed from the intestine (Berghman et al., 2005); their effect is entirely within the gut.

One of the most promising means of antibody production is through vaccination of laying hens with antigens of particular relevance to poultry production. The hens mount an antibody response, and pass on maternal antibodies into the eggs. The eggs are then processed commercially, and the antibodies removed and purified. The remainder of the product can be used in the liquid egg market. Not only are the hens producing food, but a high-value byproduct (Cook, 2004). Purified antibodies can be added to the feed of other birds to offer protection against the specific pathogen(s) against which the hen was immunized (Berghman et al., 2005; Cook, 2004). Recent research has focused on the production of antibodies to specific molecules involved in inflammation such as neuropeptides and phospholipase A<sub>2</sub> (Cook, 2004).

Exogenous antibodies are advantageous in that the product is natural, and development of microbial resistance is unlikely. A potential limitation, however, is that the maternal antibodies produced will be specific for the antigens to which the hens have been exposed. Exogenous antibodies will offer no protection to novel, unrelated pathogens. From a practical standpoint, exogenous antibodies may be more applicable when specific pathogens are expected to be encountered, or to reduce the risk of specific food-borne human pathogens.

## Conclusions

The avian immune system has innate and acquired aspects that function together to protect the bird from exposure to foreign material and microbes. Activation of the immune system often decreases performance of modern poultry. Therefore, to maximize production efficiency, it is likely beneficial that the immune system be kept in surveillance mode unless activation is required. Once activated, a rapid resolution or transition from innate to acquired immune responses is desirable to minimize the loss of productivity. The gastrointestinal tract is a major site of interaction between the bird and pathogens. The gut-associated lymphoid tissue (GALT) has aspects of both the innate (non-specific) and acquired (specific) arms of the immune system, and has a major role in minimizing access of pathogens to the bird. Manipulation of the immune system, may allow new, non-antibiotic means of maintaining productivity and safety of poultry as food products.

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# Organic versus Inorganic Trace Minerals in Poultry: The Zinc Experience

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Trace minerals are required for the normal functioning of all biochemical processes in animal body. However, the trace mineral requirements of poultry are not well defined. Commercial diets typically supplement inorganic trace minerals at a rate much higher than those recommended by National Research Council (Lesson, 2005). The ever-increasing poultry genetic potential and environmental concerns have caused the industry to reconsider how to efficiently maximize productivity while minimizing waste disposal. Research studies have shown that one way to attain these industry goals for trace mineral usage is through the use of organic trace minerals.

According to the definitions provided by the Association of American Feed Control Officials, there are currently six classes of commercially available organic trace minerals (Table 1; AAFCO, 2005).

**Table 1. Definitions of organic trace minerals (AAFCO, 2005)**

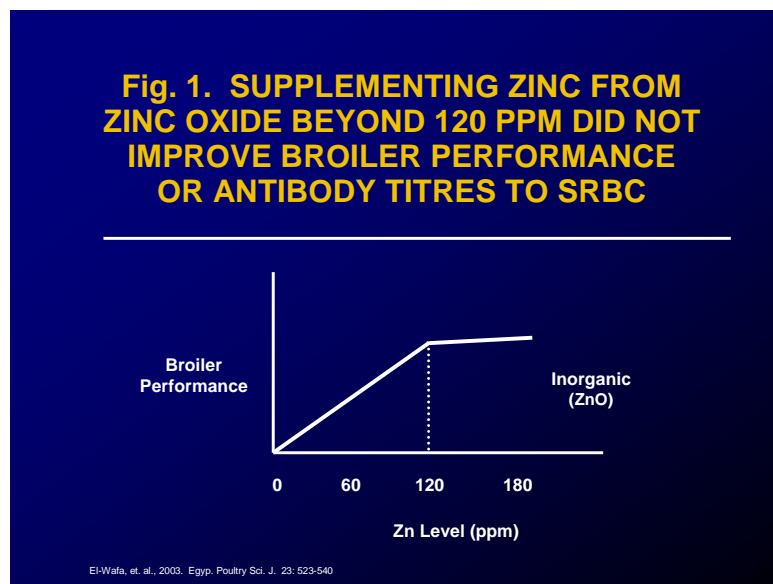
1	<b>Metal (specific amino acid) Complex</b> Product resulting from complexing a soluble metal salt with a specific amino acid. Minimum metal content must be declared. When use as a commercial feed ingredient, it must be declared as a specific metal, specific amino acid complex.
2	<b>Metal Amino Acid Complex</b> Product resulting from complexing a soluble metal salt with an amino acid(s). Minimum metal content must be declared. When use as a commercial feed ingredient, it must be declared as a specific metal amino acid complex.
3	<b>Metal Amino Acid Chelate</b> Product resulting from the reaction of a metal ion from a soluble metal salt with amino acids with a molar ratio of one mole of metal to one to three (preferably two) moles of amino acids to form coordinate covalent bonds. The average weight of the hydrolyzed amino acids must be approximately 150 and the resulting molecular weight of the chelate must not exceed 800. Minimum metal content must be declared. When used as a commercial feed ingredient, it must be declared as a specific metal amino acid chelate.
4	<b>Metal Proteinate</b> Product resulting from the chelation of a soluble salt with amino acids and/or partially hydrolyzed protein. It must be declared as an ingredient as the specific metal proteinate.
5	<b>Metal Polysaccharide Complex</b> Product resulting from complexing a soluble salt with a polysaccharide solution declared as an ingredient as the specific metal complex
6	<b>Metal Propionate</b> Product resulting from the reaction of a metal salt with propionic acid. The metal propionates are prepared with an excess of propionic acid, at an appropriate stoichiometric ratio. It must be declared as an ingredient of the specific metal propionate

However, not all organic trace minerals are created equal. Gou *et al.* (2001) showed that the solubility of various commercially available organic copper sources, including those from the same class of organic copper, was different. The authors reported that for a few

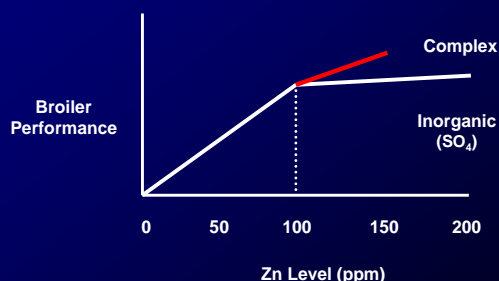
organic copper sources with high copper solubility, a large percentage of their ligands (the nitrogen portion) were not present in the solution with the copper ions. Therefore a high percentage of the soluble copper actually existed in an unbound state. This was also shown using a copper specific ion electrode. The data clearly showed that copper proteinate from source A may not be the same as copper proteinate from source B.

It is generally accepted that bioavailability of trace minerals from organic trace mineral sources was higher than that of the inorganic sources. Since organic trace mineral sources are different (via AAFCO definitions), not all organic trace minerals are the same. However, the impact of the presence of dietary antagonists on the bioavailability of trace minerals was not well documented. Wedekind *et al.* (1992) compared the bioavailability of ZINPRO<sup>®</sup> zinc-methionine to feed grade zinc sulfate using purified (crystalline amino acid), semi-purified (soy isolate), and complex (corn-soybean meal) diets. ZINPRO zinc methionine was reported to show higher bioavailability in all three diet types. The relative bioavailability of Zinpro zinc methionine compared to zinc sulfate increased (117, 177 and 206%, respectively) as complexity of the diet increased. Similarly, Wedekind *et al.* (1994) reported that when the calcium level in the test diet increased from 0.60 to 0.74%, the bioavailability of zinc from ZINPRO zinc-methionine relative to zinc sulfate increased from 166 to 292%.

Organic trace minerals were good vehicles to supply broilers with more trace minerals without increasing the dietary trace mineral levels. Adding more than 120 ppm of Zn from zinc oxide to the broiler diets did not further improve market-age broiler weight, feed conversion, and antibody titers to SRBC (El-Wafa *et al.*, 2003; Figure 1). If one assumed the bioavailability of zinc oxide was 65% relative to zinc sulfate, then, the “saturation point” for inorganic zinc for this study was equivalent to 78 ppm of Zn from zinc sulfate. Pooled data from two large scale broiler studies (Zinpro, unpublished data; Figure 2) showed that supplementing broiler diets with zinc from zinc sulfate beyond 100 ppm did not further enhance market-age broiler weight, feed conversion or livability. However, adding ZINPRO zinc-methionine beyond the 100 ppm Zn “saturation point” continued to improve broiler performance.



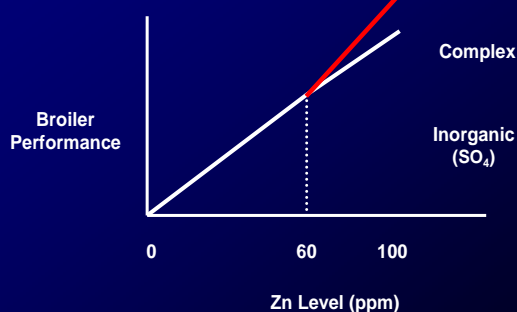
**Fig. 2. EFFECT OF COMPLEXED ZINC  
ADDED ON TOP OF ZINC SULFATE  
ON BROILER PERFORMANCE**



Zinpro, unpublished data

It was important to note that supplementing organic zinc to the broiler diets with inorganic zinc well below these “saturation points” was still highly beneficial. Two separate broiler trials were conducted (Zinpro, unpublished data) with a basal diet containing 60 ppm added zinc from zinc sulfate. Treatments involved (1) basal + 40 ppm zinc from zinc sulfate, and (2) basal + 40 ppm zinc from zinc amino acid complex. Broilers were reared to a final body weight of 8.3 to 8.5 lb. Pooled data showed that broilers fed diets supplemented with zinc from zinc amino acid complex outperformed those fed zinc sulfate in body weight, feed conversion, livability and breast meat yield.

**Fig. 3. EFFECT OF COMPLEXED ZINC  
ADDED ON TOP OF ZINC SULFATE  
ON BROILER PERFORMANCE**



Zinpro, unpublished data

Leeson (2005) reported a caged broiler study where the diets were formulated with mineral sulfates to furnish 100, 90, 30 and 5 ppm of zinc, manganese, iron and copper, respectively. These sulfates were assumed to be 70% bioavailable (“bioavailable” zinc, manganese, iron and copper were assumed to be 70, 63, 21, and 3.5 ppm, respectively). Five additional treatments involved feeding diets with a proteinate as sole mineral source that furnished 100, 80, 60, 40 and 20% of the “bioavailable” level of the mineral sulfates. There were no statistical differences among treatments for 42-day body weight and feed conversion. In a follow-up floor pen study, that involved the same inorganic control and the same proteinate used as sole mineral source to furnish either 14 or 7% of the “bioavailable” minerals (Leeson, 2005), no statistical differences were detected among treatments for 20 - 41 day weight gain, 0 - 20 as well as 20 - 41 day feed conversion. The author stated that when the proteinate was used as the sole mineral source to furnish 14% of the “bioavailable” minerals in the broiler diets, the cost was comparable to the inorganic premix used in the control treatment. Due to the ultra low mineral levels, it would be risky to feed such diets to the broilers in the field. Nonetheless, the author calculated that the zinc, iron, and copper output in the manure would be significantly reduced if any of these proteinate diets were fed to the broilers (Leeson, 2005). However, in general output will always decrease as supplementation level decreases. Feeding zinc amino acid complex had been reported to decrease broiler zinc excretion (Burrell, *et al.*, 2004).

In a series of broiler studies, broilers fed diets supplemented with 40 ppm of Zn from zinc amino acid complex on top of the inorganic control (range of added inorganic zinc: 80 - 110 ppm) were reported to improve body weight, feed conversion, livability and breast meat yield (Zinpro, unpublished data).

The macrophage tumoricidal activity of 5 week-old tom turkeys fed an organic trace mineral diet (40 ppm Zn from ZINPRO zinc methionine, 40 ppm Mn from MANPRO<sup>®</sup> manganese methionine, 80 ppm Zn from zinc sulfate and 120 ppm Mn from manganese sulfate), was reported to be significantly higher ( $P < 0.05$ ) than that of tom turkeys fed a sulfate control diet containing 120 ppm Zn and 160 ppm Mn (Ferket, 1992).

Broilers fed an organic trace mineral diet containing 40 ppm Zn from ZINPRO zinc methionine and 40 ppm Mn from MANPRO manganese methionine showed significantly ( $P < 0.05$ ) less skin tears and scratches than those fed an iso-zinc, iso-manganese and iso-methionine diet (100 ppm Zn and 105 ppm Mn from sulfates; Zinpro, unpublished data).

Rapp *et al.* (2001) gave broilers a very mild coccidial challenge using mixed field isolates. When anticoccidial was removed from the diet at 35 days of age, birds fed 40 ppm Zn from zinc amino acid complex were reported to significantly ( $P < 0.05$ ) reduce the intestinal lesion scores at day 42 when compared to those fed the iso-zinc sulfate control (115 ppm Zn).

Recently, broilers fed a diet supplemented with 40 ppm Zn from zinc amino acid complex on top of 100 ppm of Zn from zinc sulfate control were reported to significantly ( $P < 0.05$ ) increase the intestine elasticity (Zinpro, unpublished data).

Hudson *et al.* (2004) fed broiler breeders either a sulfate control diet containing 160 ppm Zn, or an iso-zinc diet where 80 ppm added Zn was furnished by zinc amino acid complex. These authors reported that broiler breeders fed zinc amino acid complex

produced 2.3 extra chicks per hen housed when compared to those fed the sulfate control. The livability of chicks produced from broiler breeders fed zinc amino acid complex was significantly ( $P < 0.05$ ) higher than those fed only the sulfates (Virden *et al.*, 2002). Chicks from broiler breeders fed a diet supplemented with 40 ppm of Zn from ZINPRO zinc methionine showed significantly heavier dry bone weight as well as enhanced cellular immune response ( $P < 0.05$ ; Kidd *et al.*, 1992, 1993). Broiler breeders fed similar diet supplemented with 40 ppm of Zn from an oxide source did not show similar responses. In fact, the responses of the oxide supplemented group were no different than the non-supplemented basal control ( $P > 0.05$ ; Kidd *et al.*, 1992, 1993).

## SUMMARY

There are currently six classes of organic trace minerals as defined by Association of American Feed Control Officials. Cross-classes or intra-class comparison of data is difficult due to differences in ligands and manufacturing processes. The best criteria to judge an organic trace mineral source is to look at the repeatability in animal efficacy data. Repeatability seen across many animal species is the best. Broiler data clearly shown that supplementing inorganic zinc beyond 80-100 ppm will not further improve broiler performance. However, supplementing selected organic trace minerals either above or below this “saturation point” has been reported to improve performance. Besides improving weight gain, feed conversion, livability and breast meat yield, supplementing selected organic trace minerals (alone or together with inorganic sources) has been reported to decrease minerals in the manure, enhance immune status, maintain skin integrity, decrease intestinal lesions due to mild coccidial challenge, increase intestine elasticity, improve breeder-progeny performance, etc.

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# Use of Biotechnology in Nutrition Research

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Classic animal nutrition research has involved the determination of nutritional requirements, diet formulation, and the monitoring of animal performance. Historically, specific nutrition research often involved the assessment of targeted pathways such as carbohydrate or lipid metabolism where biochemical and enzyme assays monitored the effects of diet. More recently with the development of the field of molecular biology, researchers have been able to study the impact of diet on the organism at the molecular level. The focus of this review is to summarize new research frontiers and to highlight the information biotechnology can provide to nutrition research.

The term “nutrigenomics” was coined in 2002 to describe the trend in human nutrition research towards individualized dietary formulation based on how diet effected the expression of specific genes and the development of specific diseases including diabetes, obesity, and heart disease. In reality the scope of this field is significantly larger.

The interaction of an organism with its diet (or nutrition source) is an intimate and complex physiologic affair that is based on multiple organ systems working in concert. The regulation of these processes can be found at all levels from genetics, gene expression, proteins, to specific metabolites. Only until recently has the technology become available to follow the regulation of these processes. Nutrition researchers are just beginning to utilize tools to ask scientific questions about diet involving genomics, functional genomics (gene expression), proteomics, and matabolomics. This review will address the capabilities and limitations of these technologies and the potential impact of a systems-wide approach on the development of nutritional science.

## **Introduction**

Dietary components (nutrients) were long thought of as a source of energy or as cofactors until the discovery of metabolites role in the regulation of enzymatic activity through allosteric control. Metabolites were also shown to regulate the secretion of hormones. Later these effects were found to be the result of modified gene expression. Biotechnology has provided evidence of two key concepts involving the interaction of diet (nutrition) and the individual organism. First, nutrition affects the individual through the modulation of expression of genetic information in response to diet composition. Second, nutritional effects must be characterized on the basis of the genetics of the individual organism.

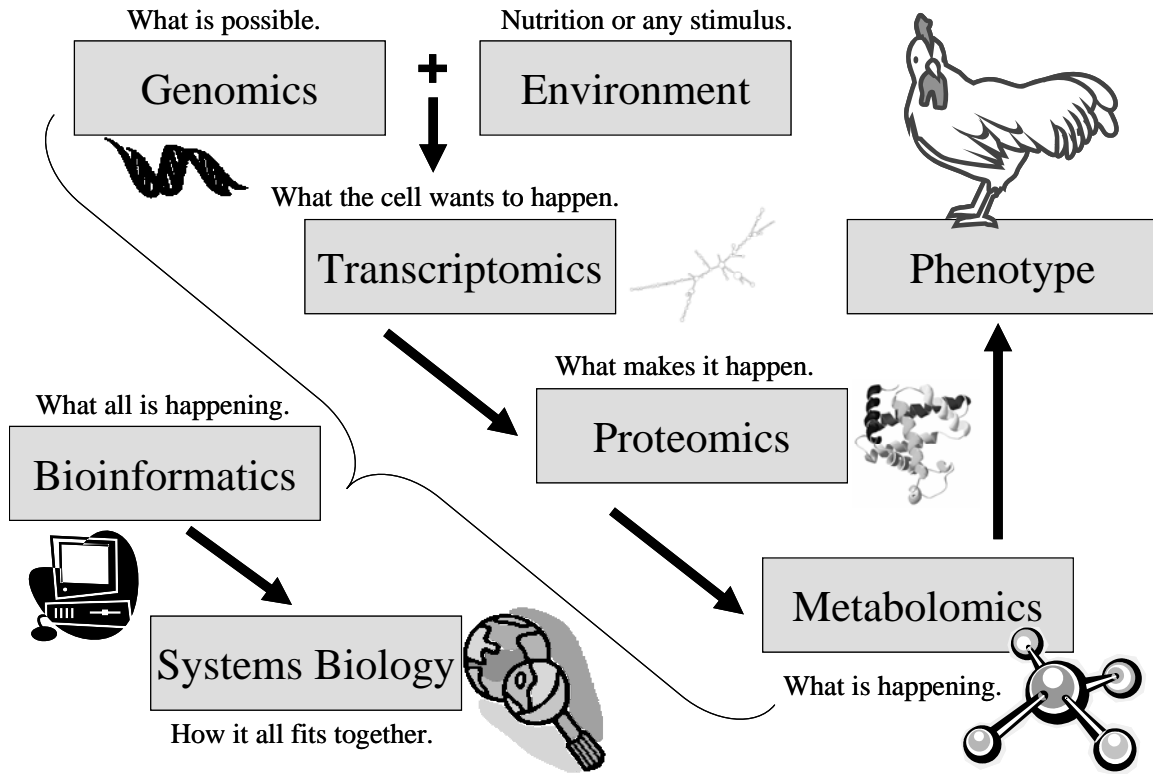
Adaptation to dietary components by the organism implies the regulation of physiologic and metabolic pathways. Most of our knowledge on the effects of nutrients on gene expression has been acquired in animal models, many examples of which can be found in a recent overview of the mechanisms by which nutrients interact with their molecular targets to modify gene expression (Muller and Kersten, 2003).

Variation of the individual response to diet can be explained by the underlying differences in genetics across a population. This observation holds true in human as well as most outbred animal populations. This variation or polymorphism is the basis for individuality and results from sequence differences within the genome of an organism. The effects of each varying sequence are minute but the culmination of the differences across the entire genome produces an individual's genotype or genetic potential. This genotype interacts with environmental factors to produce the phenotype or outward appearance or performance of an individual. Numerous studies in animals and humans have shown that individual genotypic variations can alter nutrient metabolism, from relatively mild conditions like lactase gene polymorphisms that result in lactose intolerance to potentially severe pathological conditions like phenylketonuria (Harvey et al 1998).

It is clear that the quantity and quality of the diet modulates the expression of numerous genes in various tissues. Each individual will respond to a specific diet in a unique manner concomitant with their genetic profile of polymorphisms. One of the goals of nutrigenomics research is the development of consensus responses to specific dietary stimuli so that anomalies can be identified and studied further. It is these anomalies that will provide the basis for understanding how genetic differences are associated with specific response to nutrients, how genetic differences in combination with diet result in maladies like obesity, and how specific nutrient have beneficial or deleterious effects.

Research using the knowledge from the Human Genome Project will ultimately enable scientists to understand the functions of human genes and how they are regulated. This knowledge will provide them with information on how genes and nutrients interact and the effect of individual genetic differences on diet and nutrition. This research will be directly applicable to other species whose genome sequencing projects are underway including many livestock species. Research can help to identify these effects and help to understand why certain nutrients and foods are of benefit to health. Developing within this genome era were technologies that were increasingly broad in scope that included automation, high throughput, and data intensive. Many of these technologies also involved miniaturization of standard techniques to suit the new high throughput experimental designs. A description of the state of these technologies and their implications on nutrition research follows including the study of genomics (polymorphism), functional genomics (gene expression), proteomics (protein expression), metabolomics (metabolite profiles), bioinformatics (data storage and mining), and systems biology (integrated data analysis). The organization of these processes and data sources is found in Figure 1.

**Figure 1: Diagram of the integration of molecular technologies and the flow of genetic information from the genome by its interaction with the environment through gene expression (transcription), protein expression (translation), and metabolites to the ultimate phenotype. The accumulation of information (bioinformatics) and its explanation (systems biology) are also included.**



## Genomics

Genomics concerns the analysis of DNA, the genome, and focuses on identifying the variation in the genome between individuals. Genomics tries to correlate this variation with phenotypic parameters (linkage and association studies). Variation in the sequence at the nucleotide level (substitution and small deletions, duplications, insertions) has long been considered most important; variant coding sequences directly translate to variant function and variation can be used for linkage and association studies. New quantitative technologies including array-CGH (comparative genomic hybridization) and quantitative SNP technology revealed an unexpected and large-scale copy number variation (CNV, deletions and duplications) in the human genome (Iafra et al 2004, Sebat et al 2004). The majority of CNVs include genes, thereby directly influencing the expression level (in theory 50% up or down), and should be considered as an important and previously neglected type of variation in the human genome. For measurement, SNPs (single nucleotide polymorphisms) are currently the most popular tools, although di-nucleotide repeats (tandem repeats of two nucleotides such as CA or AT) and AFLP (amplified fragment length polymorphisms, mainly employed in plants) are also widely applied. Variation and recombination in the genome is not spaced randomly and efforts currently are attempting to characterize the nature of these “hot-spots”. Currently high-throughput

genomewide analysis is facilitated using several technologies, the most powerful being array technology (DNA chips and micro-arrays), mass spectrometry (e.g. MALDI-TOF) and beads-based flow sorting. The maximal capacity lies in the order of 100,000 SNP typings per day. The current major problem is not technological but financial, with the present cost per genotype of \$0.01-0.05.

## **Gene Expression- Transcriptomics**

Focusing on the analysis of RNA (the transcriptome), transcriptomics aims at measuring the level of expression of all or a selected subset of genes based on the amount of RNA present in a sample. Currently, the most powerful tool available is DNA array technology. Using one array the expression level of up to 50,000 transcripts can be measured in parallel, and tens of samples can be screened per day. In these studies, hundreds of genes are usually varying in expression. The difficulty is to organize the results in such a way that they can be used to elucidate biological mechanisms, or to derive biological markers for a given physiological situation. Such data treatment is obviously an essential requirement if one wants to understand the overall consequences of nutrient intake.

The main limitation lies in the sensitivity of the assay as well as in data analysis. Statistically significant measurements can sometimes only be obtained for the most abundantly expressed genes, and when expression differences are changed by a factor of two or more. When smaller changes need to be detected, the measurement has to be repeated several times, making studies rather costly.

As for any new technology, array-based transcriptomics is hindered by initial limitations in analytical precision and standardization. The standardization issue was noted at an early stage. The Microarray Gene Expression Database (MGED) group was founded in 1999 with the goal of facilitating the adoption of standards for DNA array experiment annotation and data representation, as well as the introduction of standard experimental controls and data normalization methods ([www.mged.org](http://www.mged.org)). In addition, high per-analysis cost seriously reduces the number of measurements performed per study. Furthermore, different platforms are used, e.g. cDNA vs. oligonucleotide array and printed microarrays vs. on chip synthesis, and designs are regularly modified to incorporate new genes and improved probe sequences, thereby complicating data comparison (Nimgaonkar et al 2003). Several commercial suppliers produce off-the-shelf arrays (e.g. Affymetrix, Agilent, Amersham) or oligonucleotide collections for custom spotting (e.g. Illumina, Operon). Intrinsically, these provide a first 'standardization' which is desperately needed to be able to compare results from different studies. Because of these problems, studies that adequately meet rigid statistical requirement are in fact relatively scarce (Kothapalli et al 2003). In view of these weaknesses, data from array-based transcriptomics need to be interpreted cautiously.

Finally, it should not be underestimated that the major source of variability arises from the starting biological material itself. Ensuring the validity of this material, collecting all possible variables (from genome, to age and environment) and controlling the sampling conditions and timing are essential to obtain meaningful data. In this case, the use of

inbred lines is valuable in controlling the genetic variation. Another limitation lies in the tools used for data analysis (software). Current bioinformatic tools are somewhat effective but opinions vary considerably regarding the best computational algorithms to apply.

## **Proteomics**

Proteomics technology focuses on the analysis of proteins and their interactions. The challenge lies in the development of technologies, which are able to cope with the huge differences in chemical properties of proteins as well as the wide dynamic range of protein concentrations. Initially, two-dimensional (2D) gel electrophoresis was used to measure the expression level of a large number of proteins. If the full set of proteins separated by 2D gels is to be identified, automated equipment is used for excising protein spots, digesting the proteins therein, and analyzing the resulting peptides using mass spectrometry. However, 2D-gel analysis is biased towards the most abundant changes, which might lead to erroneous conclusions since also subtle variations may lead to important changes in metabolic pathways. In addition, low abundant proteins and very hydrophobic, acidic or basic proteins are often not detected and identification of the proteins resolved is time consuming and costly (Gygi et al 2000). Like with array technology, 2D-gel analysis can be combined with two-color fluorescent labeling, highlighting those proteins that differ in expression between the two samples.

Recently, mass spectrometry (MS) has come into play as an exciting and very powerful analytical tool. Proteins are submitted to proteolytic degradation to form collections of peptides that are subsequently analyzed using MS (peptide mass fingerprints). When the molecular mass of certain peptides does not concur with published structures, these peptides are further characterized by MS-MS tandem mass spectroscopy. Such analyses will also reveal post-translational modifications of proteins, such as phosphorylation. MS can be used to quickly determine the identity of a specific protein and it facilitates analysis of very complex protein samples, zooming in on those proteins that differ in expression. Impressive studies have been performed in the area of cancer research and diagnosis using mass spectrometry in combination with 2D liquid chromatography (2D-LC). The limiting step for this technology lies mainly in data analysis, i.e. computing power and the lack of adequate software tools. Generating MS traces is a matter of seconds with cost of the measurement being a minor issue. Another approach uses isotope coding and subsequent quantification relying on digestion of the protein mixture and separation on peptide rather than on protein level (shotgun proteomics). While gel-associated drawbacks are circumvented, isotope coding is limited by the risk of insufficient yields and the alteration of the sample composition. Moreover, it is often compromised by a protein bias due to tagging post-digestion, amino acid-targeted reagents and possible chromatographic separation of the light and heavy labels.

Major challenges for the field of proteomics are posed by the study of protein-protein interactions and the relationship between protein polymorphisms – nutritional value. To study protein-protein interactions, two-hybrid cloning systems have proven to be efficient and successful techniques (Fields and Song 1998). Recently, two-hybrid arrays have been developed, in which the screening is performed in a colony array format with each colony

expressing a different pair of proteins (Cagney et al 2000). Array screens can be easily automated facilitating high-throughput and reproducible protein-protein interaction screens. Furthermore, comparing the results from several assays circumvents the problem that single assays generate a high number of false positives.

Most biological questions require the differential analysis of two biological states, typically case vs. control, and, consequently, quantitative tools. It should be noted that while quantitative transcriptomics have evolved into commercialized and partially standardized platforms, quantitative proteomics is just emerging and standards are largely unavailable.

Proteins in body fluids like milk are translated from mRNAs that are expressed in different tissues, such as mammary epithelial cells and milk leukocytes. Consequently, milk proteins cannot be readily deduced from transcriptomics, and proteomics has particular relevance here. Applications include identification of minor milk proteins with potent biological function, such as growth factors, and investigation of milk protein polymorphisms. Such polymorphisms have implications for the properties and processing of milk, as well as for its nutritional value, which is determined not only by amino acid composition but also by digestibility and digestion rate of proteins (Fitzgerald et al 2003). Moreover, polymorphism may change the pattern of peptides released during digestion in the gastrointestinal tract, which may result in differences in biological activity and allergenicity of their peptide mixes.

## **Metabolomics**

Metabolomics technology focuses on the analysis of metabolites, the metabolome. It tries to measure the level of all substances (other than DNA, RNA or protein) present in a sample; the metabolome comprises the complete set of metabolites synthesized by a biological system. Such a system can be defined by level of biological organization, such as organism, organ, tissue, cell, or cell compartment levels. Today, the best tools for metabolomics research are proton nuclear magnetic resonance (NMR) and mass spectrometry (MS). Biologically relevant samples can easily be obtained from blood, sweat, urine, and feces.

Metabolomic analyses have only just begun to be in wide use. NMR-based metabolite profiling through highly quantitative and broad-spectrum classes still suffers from inherent sensitivity issues. The dynamic range is between few nanomoles to few hundred micromoles. Thus most abundant metabolites, i.e. steady-state concentrations are easily observed. However, recent cryoprobe technology shows great promise to overcome sensitivity hurdles. LC-MS, complementary to NMR, offers superb sensitivity but is limited by the essentially nonquantitative nature of mass spectrometry requiring internal standardization. Addressing automatic spectra processing, a few obstacles are worth mentioning: although peak-recognition software is getting smarter (for NMR and MS), inconsistencies in chemical shifts and baseline shifts in NMR spectra have to be compensated for and this is not a standardized task.

Publicly available metabolomic databases are being developed similarly to genomic sequence repositories and technical advances as well as improved data mining and

analysis tools are in development. Due to pleiotropic effects, the effect of a nutrient may lead to changes of metabolite levels in various, seemingly unrelated biochemical pathways. Therefore, a comprehensive analysis of all metabolites is required to understand such hidden relationships. Both sample preparation and data acquisition must aim at identifying all classes of compounds, assuring high recovery as well as experimental robustness and reproducibility. The novelty of these approaches is evident since the first meeting of the Metabolomics Society was convened in June of 2005.

## **Bioinformatics and Data Mining**

Bioinformatics is the technology enabling the data processing, clustering, dynamics, integration and storage of the overwhelmingly complex data sets produced by modern molecular research. Bioinformatics will play a crucial role to condense the massive amounts of data generated through high-through-put experimental procedures and to integrate these with data obtained from traditional techniques. The challenge is to combine all pieces of information so that all data can be looked at in a coherent way. Novel algorithms, software and hardware are being developed to translate sets of gene, protein and metabolite data into biochemical pathways.

In order to gain full access to these emerging powerful tools, it is paramount to address the enormous challenge of unifying complex and dissimilar data (Brazma, 2001). The incorporation of observations from numerous sources and domains into a unified, seamlessly searchable database and turning in it into knowledge will impact every facet of modern nutrition (Desiere et al 2002). Two important features are required in order to integrate data between databases; they have to speak the same language, and use the same identifiers for the same object. In other words, the biological domain should be described using specific vocabularies and ontologies ([www.ebi.ac.uk/GOA/project.html](http://www.ebi.ac.uk/GOA/project.html)) to allow meaningful data comparisons. The gene ontology consortium has developed a dynamic, structured, and precisely defined vocabulary for describing the roles of genes and their products in any organism. The goal of this effort is to address the deficits of the current rather divergent nomenclature schemes (Ashburner et al 2003). One of the best known providers of a human genome gene index and gene annotation, the Ensembl project ([www.ensembl.org](http://www.ensembl.org)) is an entirely Open Source project and has been widely adopted by academic and commercial organizations (Hubbard et al 2002).

Major challenges that remain to be addressed are to define ‘normal’ and ‘healthy’ versus ‘unhealthy’ profiles, especially in the pre-disease stage. Sampling location and timing will have to be optimized within ethical and practical constraints. In outbred populations like humans, detection of subtle effects may require numbers of participants that are too large to be realistic, unless participants can be pre-selected based on their genotype.

## **Systems Biology**

By helping to understand the interaction between nutrients and molecules in our bodies, the implementation of molecular biology and biochemistry in ‘classical nutrition’ research, followed by the technological revolution of molecular technologies described above, will greatly affect nutritional sciences. The first studies that span the levels of genome, transcriptome, proteome, and metabolome demonstrate this impact. Most of

these studies investigate differential effects on the level of (metabolic) pathways, and provide new mechanistic insights. However, the real potential of technologies does not limit itself to such differential display type of strategies, where the measurement of a very large set of parameters is exploited only for those parameters that show dramatic differences. The complete dataset contains significantly more information.

On top of that, the various ‘layers’ of each technology platform are of course related (genes encode RNA, which encodes the enzymes that catalyze the conversion of metabolites). Thus, in combining the datasets of genome, transcriptome, proteome and metabolome, a wealth of added information becomes available. In fact, this combination of datasets paves the way to a complete description of the biological behavior of a cellular system, in response to external stimuli.

Although the complexity of this proposed integration (i.e. systems biology) is exceeding the current bioinformatics tools and capacities, its implications for nutritional research can be enormous. Unlike biomedical interventions (drug therapy), nutrition is chronic, constantly varying, and composed of a very large amount of known and unknown bioactive compounds. Furthermore, nutrition touches the core of metabolism by supplying the vast majority of ingredients (both macro- and micronutrients) for maintaining metabolic homeostasis. This homeostasis stretches from gene expression to metabolism and from signaling molecules to enzyme cofactors. Thus, nutrition by its nature *needs* to be studied in an integrated way. So far, most of the tools for this integration were lacking, thus maintaining an unbridgeable gap between classical nutrition (studying human physiology with excursions into biochemical pathways) and biomedical sciences (elucidation disease-related molecular mechanisms). In applying systems biology to nutritional science, these paradoxical extremes are bridged and the complexity of the relationship between nutrition and health can be met by the complexity of the integrated approach. At the moment this is little more than a dream, since only premature and pragmatic example studies of this concept are currently being performed. Many hurdles need to be taken, most of them in the field of bioinformatics, before this discipline matures.

In the relation between nutrition and health (unlike the relation between nutrition and disease) it is necessary to develop a new concept of biomarker. It needs to reflect subtle changes in homeostasis and the efforts of the body (cellular systems, organs, and inter-organ interactions) to maintain this homeostasis. Also, it preferably should include a wide variety of biological actions. Furthermore, both efficacy and safety aspects should be monitored simultaneously. Single nutrients may have multiple known and unknown biochemical targets and physiological actions, which may not be easily addressed with classical biomarkers (i.e. the ‘single-gene, protein or metabolite’ approach, usually at non-physiological conditions). In addition, the efficacy assessment of health effects of nutritional components is even further complicated by the fact that single dietary constituents are hardly consumed as separate entities but are part of a dietary mixture.

The inter-individual variation at the genetic level, as discussed above, appears to be adding even more complexity to the nutrigenomics picture. Quite a number of these genetic polymorphisms have been described from a clinical genetics point of view,

simply because they pre-dispose to a pathological condition. These usually are the monogenic forms, with a pronounced effect on functionality. Given the high number of SNPs present in the human genome (13 million), it is obvious that many will be identified that could effect the relationship between nutrition and health. Instead of pursuing pathological leads, in particular the effects of these ‘minor’ variants must be studied. Establishing their impact on health, in relation to nutrition, is achieved through cohort-type studies. However, such approaches will fail simply due to a loss of power when multiple minor genetic polymorphisms are involved, all acting on the same physiological process. Nutrition research should not ignore these minor variants, because several subtle changes may together produce large effects, e.g. obesity can both be the result of one pronounced SNP as well as an interplay of many less pronounced variants.

“Nutri-biomics” may contribute in this effort, by describing the mechanisms in terms of kinetic and dynamic models, and verification and validation of these models with genotyping combined with functional analysis on the level of RNA, protein and metabolites.

## **Conclusions**

State-of-the-art technologies are being used to study the effect of dietary habits on health promotion and disease prevention. Not all individuals respond identically to dietary interventions, and meaningful biological interpretation of the generated data is a very complex issue. Intense collaboration between biologists, analytical scientists, statisticians and bioinformaticians is essential in order to gain the most from this new generation of data. To generate interpretable results one must start with a clear and solid question. The amount of data available in public or proprietary databases is increasing rapidly, underlining the central role of bioinformatics to transform raw data into relevant biological knowledge. Furthermore, use of multiple experimental tools and methods will increase the reliability of the results. Advancement of research in computational and analytical science will gradually transform nutrition into a more systematic and hypothesis-driven science. To accelerate and coordinate successful application of high throughput technologies in the field of nutritional research, collaborative actions and networks will be necessary. Already institutions in the U.S. and abroad have developed programs and organizations to address the impact of nutrition on biological systems in a coordinated effort. Only in this manner can the true interaction between an organism and its diet be ascertained.

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