INTRODUCTION

By 2050, the population is expected to increase to more than 9 billion people; as a result of this anticipated growth, the demand for high-protein animal meat products is expected to increase anywhere from 70 to 100%. Global chicken production has already increased 4-fold between 1960 and 2010 (Godfray et al., 2010); in addition, the USDA Economic Research Service indicates that the US poultry industry, which is one of the largest food production industries in the nation, already generates an annual revenue that exceeds 45 billion dollars. Therefore, poultry sectors including industry, government, and academia are confronted with a new array of challenges, such as global food security, climate change, emerging infectious diseases, regulatory ban of antimicrobials, high-intensity production conditions, and waste management. Furthermore, agricultural animal scientists need to consider addressing these challenges using modern research tools and should develop sustainable agricultural management systems that will be compatible with environmental and consumers’ needs.

The global animal industry needs to address the increasing regulatory restrictions on the use of antibiotic growth promoters (AGP) in animal production. Many AGP have already been restricted by animal farms in the European Union (EU) and some parts of Asia. Soon, other countries are expected to be under increasing scrutiny as consumers’ concerns about drug-resistant superbugs increase. Although the rapid progress in the poultry production system that we witnessed during the last half century was partly due to the use of AGP, frequent subtherapeutic use of AGP in agriculture has raised many concerns with respect to human health due to the potential occurrence of resistance among pathogenic bacteria and parasites. These concerns first led the EU to legislatively withdraw all AGP from poultry feeds beginning January 1, 2006. This ban on AGP was further extended to include anticoccidial ionophores in 2007. Although the legislative ban on AGP has been slow in progress and may not immediately affect the poultry industry in the non-EU countries, large chicken food industries, such as McDonald’s Corporation and Kentucky Fried Chicken, have already announced to promote AGP-free poultry meats for consumers. Accordingly, scientific evidence-based publications are supporting the possibility of sustaining intensive modern farming without the use of AGP, especially in the area of disease control. It is now the responsibility of agricultural scientists to convince the poultry industry the relevance of these new scientific findings so that practical and effective alternative strategies can be developed that will mitigate the use of antibiotics.
AGP: Mode of Action and Their Potential Alternatives

There is no doubt that the AGP that have been widely used in agricultural animal production to promote the growth of animals since the late 1940s have had significant impacts on the progress of the animal industry. Now, there has been a great deal of information on new biocontrol approaches for preventing and treating bacterial, viral, and parasitic pathogens in food animal production. Multiple alternatives, including prebiotics, probiotics, phytomolecules (herbs and essential oils), hyperimmune antibodies, bacteriophages, antimicrobial peptides, and toll-like receptor (TLR) agonists, have already been used by the animal industry for various claims, but it is generally accepted that none of these alternatives are known to be as effective as AGP in field application. However, a combination of additives (e.g., probiotics and prebiotics) or novel feed additives have shown some efficacy to compensate for production loss, in the absence of AGP, with economic returns. Furthermore, the exact modes of action of many widely used AGP have not been proven. There has been a long belief that AGP mediated growth through their antimicrobial activity on gut microbiota, especially pathogens and bacteria that cause growth depression, according to Gaskins et al. (2002). This antimicrobial hypothesis of AGP has been supported by observations that showed enhanced growth of chickens raised in a polluted environment and the lack of this growth-enhancing effect under germ-free or clean environments. In contrast, Niewold (2007) recently proposed that AGP directly interact with the complex intestinal ecosystem, especially with those innate immune cells mediating inflammatory response. According to Niewold’s hypothesis, AGP enhance growth by directly acting on inflammatory immune cells making concomitant or subsequent changes in microflora that lead to an anti-inflammatory gut environment. Although the detailed mechanisms of different types of AGP need to be better investigated and identified, increasing scientific evidence supports the notion that nonantibiotic alternatives for AGP that are capable of manipulating inflammatory innate immune response can be developed.

Multiple dietary immunomodulators have been suggested as alternatives to AGP. “Immunomodulation,” according to the Merriam Webster dictionary (www.merriamwebster.com), is defined as “modulation of the immune response or the functioning of the immune system by the action of an immunomodulator.” With this in mind, immune modulators include antimicrobial peptides (AMP), TLR ligands and agonists, prebiotics, probiotics, hyperimmune antibodies, herbs and essential oils, bacteriophages, anti-infectives, and anti-virulence drugs. Furthermore, studies done on mice and humans have shown solid scientific evidence that many of these AGP alternatives, such as TLR ligands, probiotics, herbs, and essential oils, immunomodulate host immunity by directly interacting with conserved innate sensing molecules present on innate immune cells.

Innate Immunity: Primary Line of Host Defense

When host innate immune cells, such as dendritic cells, encounter foreign antigens, pathogens, or vaccines, the innate immune cells recognize various pathogen-associated molecular patterns (PAMP) that are unique microbial signatures associated with different pathogens via germ line-encoded, highly conserved, host innate immune receptors called pathogen recognition receptors (PRR). Initial binding of a PRR with a PAMP triggers a series of complex and sophisticated intra- and intercellular signaling pathways that lead to the final activation of NFκβ and inflammatory response. Different types of innate immune responses are determined by the types of PRR that are activated. For example, PRR, such as TLR, recruit a specific set of adaptor molecules that contain Toll/IL-1 receptor domain, such as MyD88 and TRIF, to initiate downstream signaling events that lead to the secretion of inflammatory cytokines, type 1 interferons, chemokines, and antimicrobial peptides. Moreover, the activation of TLR signaling leads to maturation of dendritic cells that contribute to the generation of adaptive immunity and subsequent differentiation of naïve T-helper cells into mature effector cell types with diverse functions, such as Th1, Th2, Th17 and Treg, which secrete different types of cytokines, including IFN-γ, TNF, IL-10, TGF-β, IL-4, and IL-5. Activation of innate immune cells and complex downstream signaling pathways also leads to the development of antigen-specific, long-lasting memory response that represents a secondary line of host defense called adaptive immunity. Recent studies indicate that there is a close cross-talk between innate and adaptive components of host immune system through activated receptors and secreted soluble effector molecules, and thus the type of initial immune response elicited by an immunomodulator will have profound influence on the quality of secondary line of host defense.

Immune Modulation Via Innate Immune Sensing Molecules: Some Examples

One of the initial steps triggering innate immune response involves germ-line encoded, highly conserved, innate immune sensing molecules of PRR, which include TLR, nucleotide-binding oligomerization domain proteins (NOD), retinoid-inducible gene 1, and C-lectin binding receptors. The TLR are the ligands of PAMP that recognize diverse types of pathogens, and 10 and 12 functional TLR have been identified in human and mouse, respectively, each TLR detecting distinct PAMP derived from viruses, bacteria, mycobacteria, fungi, and parasites.
**Stimulation of Innate Immunity By a Parasite-Derived TLR Ligand.** *Eimeria* parasites, the causative pathogens of coccidiosis, contain several components that are stimulatory for immune cells, and they activate innate immunity and inflammatory response. In 2004, our laboratory showed that a conserved antigen of sporozoites of *Eimeria*, profilin, is a parasite PAMP that stimulates T-lymphocytes and induces IFN-γ production. Profilin is a highly conserved TLR ligand of apicoplast parasites and has been identified in other apicoplast parasites, including *Neospora*, *Cryptosporidia*, *Toxoplasma*, and *Plasmodium*. The soluble fraction of *T. gondii* tachyzoites contains a strong inducer of IL-12 known as toxophilin, a profilin-like molecule implicated in parasite motility and invasion, and is recognized by TLR11 in mice. Although the nature of chicken TLR that *Eimeria* profilin binds has not been identified yet, our subsequent studies have demonstrated that immunization of naïve chickens with recombinant profilin protein or profilin-encoding cDNA plasmid using in ovo or intramuscular injection induced significant antigen-specific T lymphocyte proliferation response and mediated significant protection against live parasite challenge infection.

**Modulation of Innate Immunity Using Gut Commensal Bacteria.** Commensal bacteria on the intestinal mucosa contain many probiotic ligands (such as long surface appendages, polysaccharides, and lipoteichoic acids) that can communicate with PRR, inducing downstream signaling pathways that lead eventually to probiotic (health-promoting) effects. Because the final host response is determined by the coordinated action of the signals induced by the different receptors on various cell types, understanding the mode of action of different probiotics or direct-fed microbials (DFM) and their associated ligands that can modulate host innate immune response will be important if we want to apply DFM to immunomodulate host immunity. Probiotics are defined as “live microorganisms, that when included in foods can influence the composition and activity of the gut microbiota, modulate the inflammatory response, improve the nonspecific intestinal barrier, and reinforce or modulate the mucosal and the systemic immune responses.” Microorganisms frequently utilized as probiotics in poultry production include *Bacillus*, *Bifidobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Saccharomyces cerevisiae*, and *Aspergillus oryzae*. *Bacillus subtilis* has long been considered a nonpathogenic, spore-forming, soil microorganism that is an inhabitant of the gastrointestinal tracts of both vertebrates and invertebrates. Many studies demonstrated that *B. subtilis* can grow and sporulate in the gut, and they can promote the development of the gut-associated lymphoid tissues (GALT). Thus, feeding *B. subtilis* could have beneficial effects in strengthening the immune system and perhaps priming it for an adaptive immune response. In a recent study in mice, *B. subtilis* promoted active lymphocyte proliferation within the Peyer’s patches accompanied by a marked increase in the expression of several cytokines, and more interestingly, the vegetative cells of *B. subtilis* upregulated the expression of TLR2 and TLR4.

We have recently evaluated several field isolates of *B. subtilis* strains by continuous feeding of young broiler chickens with the spore-supplemented standard poultry diet to investigate the probiotic effects of *Bacillus* strains (Lee et al., 2010). Depending on the *B. subtilis* strain, feeding diets supplemented with *B. subtilis* spores increased various intestinal intraepithelial T-cell subpopulations, cytokine mRNA levels, and macrophage function. Feeding of young broiler chickens with *B. subtilis*-based DFM also enhanced NO production and phagocytosis of peripheral blood-derived macrophages. Plasma NO levels were significantly higher in groups given DFM-supplemented diets compared with the control group, and the phagocytosis of green fluorescence-labeled *Salmonella* by macrophages was significantly augmented in chicken groups on DFM-supplemented diets. Further studies of dietary *Bacillus*-based DFM effect on disease was tested using an avian coccidiosis infection model. Following an *E. maxima* challenge infection, DFM-fed chickens showed enhanced disease resistance with higher BW gain and decreased intestinal lesions compared with uninfected control birds. Detailed immune pathways that were affected by *Bacillus* treatment were further examined using a high-throughput gene expression analysis. Various immune-related genes, especially ones associated with inflammatory response, were upregulated in the gut of probiotic-treated chickens.

**Modulation of Innate Immunity Using Plant Phytochemicals.** Phytonutrients or phytochemicals are plant- or fruit-derived chemical compounds possessing health benefits, including promoting tumor killing and increased resistance to infectious diseases, and have been used as health-promoting agents by many cultures for several millennia. While numerous studies have shown disease prevention or immune enhancing effects of phytonutrients, very few reports have examined the underlying mechanisms for their specific immune modulating effects. Many phytochemicals are known for their anti-inflammatory properties, and an increasing number of studies have indicated that diets rich in anti-inflammatory phytochemicals may have beneficial effects in ameliorating tissue damages caused by pathogens. One promising new avenue to develop a drug-free disease control strategy is the use of natural foods and herbal products to reduce inflammatory effects of infections and to enhance host defense against microbial infections and tumors. This approach is based on many scientific data demonstrating the immunomodulatory effects of natural and herbal products in many agricultural animal species as well as humans. The intestinal mucosal system plays a central role in the exclusion and elimination of harmful dietary substances in humans and animals. Recent evidence revealed that certain phytochemicals, such as cinnamaldehyde and curcumin, inhibit PRR activation by targeting the receptor itself.
or the specific downstream signaling molecules. These results suggest the possibility that PRR-mediated inflammation and the consequent risk of damaging disease conditions can be suppressed by particular food supplements containing anti-inflammatory phytonutrients. Thus, identifying the molecular targets by which dietary factors modulate PRR-mediated signaling pathways and target gene expression would provide new opportunities to reduce risk and to manage inflammatory diseases associated with field infections of poultry.

Using mice as an animal model, scientific evidence for the dietary modulation of PRR-mediated proinflammation by certain phytonutrients have been shown. For example, curcumin, helenalin, and cinnamaldehyde with αβ-unsaturated carbonyl groups, or sulforaphane with an isothiocyanate group, have shown to inhibit the activation of TLR4 by interfering with cysteine residue-mediated receptor dimerization, whereas resveratrol, with no unsaturated carbonyl group, did not. Furthermore, curcumin and helenalin, but not resveratrol, also inhibited NOD2 activation by interfering with NOD2 dimerization. In contrast, resveratrol specifically inhibited TLR3 and TLR4 signaling by targeting TANK binding kinase 1 and receptor interacting protein 1 (RIP1) in Toll/IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF) complex. Together, these results show that PRR and downstream signaling components are the molecular targets for certain dietary disease prevention strategies.

Cinnamaldehyde is a constituent of cinnamon that is widely used as a flavoring compound and has been traditionally used to treat many human diseases, including dyspepsia, gastritis, and inflammatory diseases. Cinnamaldehyde was reported to possess antioxidant, antimicrobial, and larvicidal activities. Recently, in this laboratory, we have shown the effects of cinnamaldehyde on in vitro parameters of poultry immunity and in vivo protection against avian coccidiosis. In vitro, cinnamaldehyde treatment induced cell proliferation of chicken spleen lymphocytes and activated macrophages to produce higher levels of NO. In addition, broiler chickens given a diet containing cinnamaldehyde had elevated levels of IL-1β, IL-6, IL-15, and IFN-γ transcripts in intestinal lymphocytes compared with the control chickens. Feeding of broiler chickens with diets supplemented with purified cinnamaldehyde consistently enhanced innate immunity and provided enhanced protection against live parasite challenge infection, as indicated by reduced fecal oocyst shedding and enhanced BW gain. Dietary feeding of cinnamaldehyde along with other plant phytochemicals, such as carvacrol and capsicum, showed synergistic enhancement of gut innate immunity against intestinal parasitic and bacterial infections. Although the mechanisms that are responsible for these phenomena are unknown, it has been suggested that they may involve morphological modification of gastrointestinal mucosal cells or altered expression of metabolism-related genes.

Further studies in our laboratory to delineate the intestinal immune pathways affected by cinnamaldehyde treatment using a high density avian gene chip showed that dietary cinnamaldehyde treatment of young chickens changed significant intestinal gene expression levels, especially genes associated with antigen processing and peptide presentation. Cinnamaldehyde-mediated gene changes of innate immune system with observed enhancement of disease resistance against intracellular parasites could have practical application in developing dietary immune enhancing strategies to reduce antibiotics. Our current study on the investigation of plant phytochemicals, using a necrotic enteritis disease model, showed that C. perfringens-induced gut lesions were significantly reduced in broiler chickens that were fed a diet containing essential oil components compared with the C. perfringens-infected untreated control chickens.

Final Comments

Concerns over the use of AGP on increasing incidence of antibiotic resistance are driving legislative policies to restrict the use of antibiotics on animal farms worldwide. Consequently, there is a growing concern that the potential development of antibiotic-resistant strains within food-animal production facilities and among food-borne bacteria could seriously compromise current medical interventions and public health. In some countries (such as those in the EU), the use of AGP has been discontinued, and some Asian countries are beginning to follow the EU in banning AGP. With increasing consumers’ concerns about drug-resistant microbes, new strategies for prevention of animal diseases that do not promote the creation of selection pressure favoring the development of antimicrobial resistance need to be developed. These drug-free biocontrol approaches for reducing bacterial, viral, and parasitic pathogens in food-animal production may include innate immune molecules with antimicrobial function, such as antimicrobial peptides, defensins, bacteriophages, bacteriophage lysins, or other naturally occurring antibacterial lytic enzymes, such as bacteriocins, recombinant or hyperimmune therapeutic antibodies, pre- and probiotics, bioactive phytochemicals (herbal extracts and volatile oils), or other antivirulence biotherapeutic alternatives. In this regard, as shown in our recent studies, the dietary immunomodulation of gut immunity in broiler chickens using natural dietary supplements, such as TLR ligands, DFM, and plant-derived phytochemicals that interact with innate sensing molecules to stimulate innate immunity, is a promising alternative strategy that can be applied to many infectious diseases besides coccidiosis, where traditional prevention methods show limitations. Furthermore, the underlying immune mechanisms involved in various dietary strategies using TLR ligand-, DFM-, and plant phytochemical-mediated immune enhancement of innate immunity should be investigated to maximize its effect and
to develop a rational synergistic approach for disease control. Furthermore, application of high-throughput functional genomics tools in delineating detailed immune mechanisms associated with alternative disease control strategies will lead to enhanced understanding of how different alternative strategies function. There is also increasing scientific evidence that implicates certain antibiotics that disrupt the normal microflora of the gut can yield negative consequence on the innate immune system, disease resistance, and overall animal well being. As we move into the 21st century and the demands for animal food products increase to meet the nutritional needs of a growing world population, developing drug-free alternative strategies to prevent and control animal diseases is a global issue and a critical component of our long-term efforts to alleviate poverty and world hunger.

Questions from D. J. Caldwell

**Question 1.** To date, probiotics or direct-fed microbial cultures have received the greatest degree of commercial acceptance of all non-antibiotic growth promoter products discussed in this opinion paper. The original work of Nurmi and Rantala (1973) introduced the concept of feeding or administering healthy gut microflora in poultry within the overall concept of competitive exclusion for pathogen reduction and health. The basis for improved gut health or pathogen exclusion within this model, which has been supported by many investigations in poultry, is based upon the ability of the administered bacteria to 1) competitively exclude pathogens by competing for intestinal colonization attachment sites and 2) production of antimicrobial compounds by the beneficial organisms present within the culture (probiotic or direct-fed microbials) that keep pathogenic bacteria suppressed within the gut microenvironment. As such, this well-established mechanism does not necessarily involve immune stimulation. Would you please discuss the potential for non-immune-mediated mechanisms to participate in the perceived benefits you discuss in this article?

**Response 1.** Dietary probiotics positively affect food-animal production and health in diverse ways that may or may not involve the immune system. In the case of poultry, the non-immune-mediated effects can be categorized as those affecting bird performance traits (e.g., weight gain, feed intake, feed efficiency, and egg production), food quality (e.g., meat tenderness, low abdominal fat contents, and cholesterol concentration), digestive physiology (e.g., nutrient and mineral digestibility, enzyme activity), and microbial activity (e.g., ammonia content and urease activity). In addition, probiotics may promote intestinal epithelial barrier integrity, for example, by reducing normal epithelial cell apoptosis and mucosal turnover. Although the detailed non-immune-mediated mechanisms of probiotics remain to be completely determined, it is likely that multiple nonredundant processes occur. What is clear at this stage is that the gut microbial ecology of newly hatched chickens is not sufficiently matured to overcome detrimental stimuli, such as infection by enteric pathogens. As a result, an unbalanced ratio of beneficial to nonbeneficial bacteria in the intestinal tract of birds impairs growth performance and increases susceptibility to disease. Future studies are warranted to determine whether probiotics may be generally helpful in reducing common enteric avian diseases of chickens and enhancing the profitability of the poultry industry.

**Question 2.** The most significant consequence in animal health to result from the antibiotic growth promoter (AGP) ban in the European Union is a marked increase in cases of necrotic enteritis in commercially reared broilers. While you advocate for the discontinued use of AGP in poultry due to the availability of the acceptable alternatives you have reviewed, you discuss necrotic enteritis and one if its etiological agents, *Clostridium perfringens*, very little. Regarding the mechanism of action of AGP on growth promotion and enteric health in commercial poultry, one widely held opinion relates to the potential for drugs such as bacitracin and virginiamycin to consistently suppress an overgrowth or “bloom” of toxigenic *Clostridium perfringens*. It seems entirely possible that this scenario could be completely devoid of any involvement of the mucosal immune system in the chicken. Further, given the nature of *C. perfringens* in the intestinal microenvironment, very little to date has been published on how an upregulated immune response may assist the chicken in resolving a *C. perfringens*-mediated case of necrotic enteritis in the chicken gut. Please discuss how these observations may interact with the proposed immune mechanisms you discuss in this article.

**Response 2.** Necrotic enteritis (NE) is caused by *Clostridium perfringens* and is partially controlled by ionophore anticoccidial drugs, which also have antibacterial activity, and by some antibiotic growth promoters. Recently, NE has re-emerged as a significant problem as a result of restrictions on the use of in-feed antibiotics, modern practices of high-density housing conditions, and re-use of litter. Globally, the economic loss to the US poultry industry due to NE is estimated to be $2 billion annually, largely due to the cost of medical treatment and impaired growth performance. Thus, there is an urgent need to develop rational, alternative, and integrated management strategies to control NE. Infection of chickens with *C. perfringens* leads to decreased feed intake, reduced feed conversion and growth rate, feed, and flock nonuniformity. Necrotic enteritis-induced weight loss primarily results from poor nutrient absorption following intestinal damage mediated by a host inflammatory responses against *C. perfringens* toxins. Antibiotics such as bacitracin and virginiamycin suppress the growth of *C. perfringens*, thus decreasing gut inflammatory responses and reducing intestinal damage. In addition to its toxins, *C. perfringens* bacteria are recognized by the host innate immune system via their highly conserved pathogen-as-
sociated molecular patterns (PAMP). Among these are *Eimeria* profilin, an actin-binding protein, and the peptidoglycan of gram-positive bacteria such as *Clostridium*. Bacterial PAMP are recognized by chicken pattern recognition receptors (PPR). Toll-like receptors (TLR) are one type of PRR expressed on the surface of dendritic cells and other leukocytes that play a critical role in induction of innate immune and inflammatory responses against invading pathogens. More specifically, interaction of PAMP with their cognate TLR leads to activation of NF-κB and subsequent pro-inflammatory cytokine production. Coccidiosis and NE are 2 examples of enteric diseases where pathogen interaction with the host immune system elicits a severe inflammatory response that can damage the gut and cause nutrient malabsorption and BW loss. Our recent study showed that the pro-inflammatory mediators IL-8, TL1A, and LITAF were significantly increased in the gut of NE-affected birds. One of our research goals is to identify dietary strategies that decrease this destructive intestinal inflammatory response to a level that prevents tissue damage without impacting immune clearance of infective pathogens. In this regard, we are currently investigating phytochemicals, such as curcumin and cinnamaldehyde, for their anti-inflammatory effects in avian NE. Prior studies in humans and murine model systems have shown that cinnamaldehyde suppressed bacterial lipopolysaccharide-induced TLR4-mediated NF-κB activation, although the detailed molecular mechanisms have not been fully elucidated. Similarly, our recent unpublished results have demonstrated that feeding of young birds with a cinnamaldehyde-supplemented diet reduced gut inflammation and decreased the associated intestinal lesions caused by *C. perfringens* infection. Ongoing studies are directed at identifying the molecular and cellular processes through which dietary cinnamaldehyde, or other phytochemicals, may limit gut damage while still maintaining a protective host immune response.

**REFERENCES**


