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Direct-Fed Microbials and Their Impact on the Intestinal Microflora and Immune System of Chickens

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Direct-fed microbials (DFMs) are live microorganisms which confer a health benefit to the host. The mode of action of DFMs involves multiple mechanisms, including direct inhibition of enteric pathogens and indirectly through competitive exclusion of pathogens by the normal gut microbiota. Additionally, recent basic research efforts have focused on the effects of DFMs on promoting host immunity and on the complex interactions between the gut microflora and immune system development. This review will summarize the latest developments in DFM studies with particular emphasis on the underlying mechanisms of immune enhancement.

Key words: *Bacillus subtilis,* direct-fed microbials, gut microflora, immunomodulation, poultry

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Introduction

A close relationship exists between the development of the normal intestinal microbial population and resistance against enteric pathogens, and it is now well-known that the gut microflora plays a critical role in maintaining homeostasis which is critical for maintaining optimal animal health. In the case of newly hatched chickens, not only is the gastrointestinal tract (GIT) sterile, but also is relatively immuno-incompetent. Both effects combine to render chicks highly susceptible to pathogen colonization of the GIT (Nurmi and Rantala, 1973; Donoghue *et al.,* 2006). Deliberate introduction of beneficial microorganisms, such as direct-fed microbials (DFMs), or probiotics, into the GIT is commonly practiced in the poultry industry to decrease the incidence of enteric infectious diseases (Choct, 2009). Although this practice is not new, DFMs have received renewed attention in recent years as prophylactic agents against intestinal diseases by balancing the normal microfloral population and by modulatioin of host immunity (Callaway *et al.,* 2008). The effects of DFMs on animal health and food production can be quantified by several well-established parameters, including performance traits (feed intake, feed efficiency, weight gain, egg production), food quality (meat tenderness, abdominal fat content, cholesterol levels), digestive physiology (nutrient and mineral digestibility, enzyme activity), and microbial activity (ammonia content, urease activity).

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General Overview of DFMs

Originally, DFMs were defined as live microorganisms which, when administered in adequate amounts, conferred a health benefit on the host by balancing the populations of normal intestinal microorganisms (FAO/WHO, 2002). This definition has been broadened by Maldonado Galdeano *et al.* (2007) 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." Based on data retrieved from the Web of Science (2009), the number of DFM-related publications has progressively increased over the past several years: 2005 (n=570), 2006 (n=687), 2007 (n=833), 2008 (n=936), 2009 (n=716 as of September). During this time, DFM-related research has been applied to microbiology (n=841, 22.5%), food science (n=780, 20.8%), biotechnology (n=606, 16.2%), gastroenterology and hepatology (n=385, 10.3%), immunology (n=341, 9.1%), nutrition (n=319, 8.5%), and animal science

(n=275, 7.3%).

Bacteria frequently utilized as DFMs in poultry production include *Bacillus, Bifidobacterium, Enterococcus, Lactobacillus, Lactococcus,* and *Streptococcus* (Kabir, 2009). In addition to bacteria, yeast such as *Saccharomyces cerevisiae* (Zhang *et al.,* 2005) and fungi such as *Aspergillus oryzae* (Lee *et al.,* 2006) have also been used. Patterson and Burkholder (2003) reported that the ideal characteristics of DFMs were host origin, stability and viability during processing and ingestion, and activity in the GIT so as to influence the host microflora and immune system. These and additional criteria for selecting functional DFM candidates have been summarized (Kabir, 2009).

Gut Microorganisms and Immune System Development in Poultry

At hatch, the alimentary tract and immune system of chicks are less well developed compared with mature birds, which renders them susceptible to infection by enteric pathogens (Lowenthal *et al.,* 1994; Koenen *et al.,* 2002). While the small intestinal microflora of adult birds is established within 2 weeks of hatching, the adult cecal flora, which is mainly composed of obligate anaerobes, required up to 30 days to develop (Amit-Romach *et al.,* 2004). The adult GIT microflora is composed of 10^7 to 10^{11} bacteria per gram of gut contents (Apajalahti *et al.,* 2004). From molecular studies, at least 640 species representing 140 genera are present in the intestinal cecum. Of these, 10% were identified as previously known bacteria based on 16S rRNA gene sequences, while the remaining sequences belonged to unidentified organisms (Apajalahti *et al.,* 2004).

It is well-known that a close relationship exists between the GIT microflora and development and/or maintenance of a functional intestinal immune system (Salminen *et al.,* 1998; Gabriel *et al.,* 2006). For example, germ-free mammals have a higher susceptibility to intestinal infections (O'Hara and Shanahan, 2006) and are unable to mount an effective antibody response until re-establishment of their gut microflora (Rhee *et al.,* 2004). Additionally, $CD4^+$ and $CD8^+$ lymphocytes, the primary effectors of cell-mediated immunity, possess relatively naïve phenotypes in germ-free animals, but following intestinal colonization, they acquire more typical activated phenotypes (Cebra, 1999). Following hatching, chicken adaptive immunity requires at least three weeks for complete maturation and development (Beal *et al.,* 2006). In newly hatched chickens, some degree of immune resistance is established by innate immune effector mechanisms and maternal antibodies, primarily IgY transmitted from hen yolk. However, antibodies are mainly effective against extracellular pathogens and generally do not protect against intracellular microbes, such as *Eimeria* and *Salmonella* that constitute economically important poultry enteric pathogens.

Proposed Mode of Action of DFMs

The direct inhibitory effects of DFMs on enteric pathogenic bacteria is well-documented (Reid and Friendship, 2002; Hariharan *et al.,* 2004; Dahiya *et al.,* 2006; Callaway *et al.,* 2008). For example, *Bacillus* DFM strains based on primary isolates from poultry litter, swine lagoons, rumen fluids and other environments were shown to inhibit the growth of avian pathogenic *Escherchia coli* and *Clostridium perfringens* type A *in vitro* (Rehberger and Jordan-Parrott, 2005). In addition, two other mechanisms have been documented, namely maintenance of a balanced microfloral population and host immunomodulation (Erickson and Hubbard, 2000; Corthesy *et al.,* 2007; Maldonado Galdeano *et al.,* 2007; Kabir, 2009; Ng *et al.,* 2009; Yang *et al.,* 2009).

Balance of Intestinal Microflora

DFMs inhibit pathogenic microorganisms in the intestine by competitive exclusion for metabolic substrates and bacterial attachment sites to epithelia as well as the production of antimicrobial substances (Yang *et al.,* 2009). Several studies have documented that *Lactobacillus* and *Bacillus* DFMs decreased the levels of harmful enteric pathogenic bacteria and increased the levels of beneficial lactic acid producing bacteria in the normal microbiota (Table 1). Differences in DFM-induced changes in the composition of the chicken gut microbial community have been directly linked to improved performance (Torok *et al.,* 2008). While *Lactobacillus*-based DFMs required input levels of 10^5–10^7 colony forming units (cfu) per gram of diet, *Bacillus*-based DFMs were effective at 10^3–10^4 cfu/g. The DFM-mediated decrease in *E. coli* and *Clostridium*-related pathogens is noteworthy because these pathogens are responsible for diseases of high concern to the poultry industry, such as gangrenous dermatitis, necrotic enteritis, collibacillosis, and enteritis of unknown etiology (Smith and Helm, 2008). Furthermore, Chichlowski *et al.* (2007a) reported that *Lactobacillus*-based DFMs lowered the load of segmented filamentous-like bacteria (SFB) on the ileal mucosal surface, but increased the SFB population on the cecal surface, compared with DFM-free controls. SFB are non-pathogenic, Gram positive, anaerobic, spore-forming bacteria that normally inhabit the chicken GIT (Fuentes *et al.,* 2008; Shima *et al.,* 2008). In mammals, SFB are also known to enhance the expression of genes involving defensive/immune functions in the gut (Shima *et al.,* 2008), and the reported modulation of SFB levels following DFM administration in chickens warrants further studies.

DFMs also have been shown to impart beneficial effects in chickens using *in vivo* challenge studies with intestinal pathogens (Table 2). *Lactobacillus* or *Bacillus* DFMs protected chickens against experimental *Salmonella* infection by the reduction of 1–3 $\log_{10}$ cfu/g of tissue compared with DFM-free and challenged controls. Decreased feed conversion ratios and lessened mortality have also been noted. The beneficial effects of DFMs on experimental

Table 1. **Effects of DFMs on normal intestinal microflora of chickens**

DFM strain	Dosage	Age of birds	Effect	Reference
Lactobacillus	3×10^5 cfu/g of diet	21 days	Less segmented filamentous-like bacteria (SFB) in ileum	Chichlowski *et al.,* 2007a
			Dense bacterial population on cecal surface	
Lactobacillus	Not specified	49 days	12% decreased prevalence of *Campylobacter jejuni*	Willis and Reid, 2008
Lactobacillus	2×10^6 cfu[1]/g of diet	42 days	ca. 1.0 $\log_{10}$ cfu increased *Bifidobacterium* spp, *Lactobacillus* spp., and Gram positive cocci per gram of cecal digest	Mountzouris *et al.,* 2007
			No effect on total aerobes, coliforms, total anaerobes, or *Bacterodies* spp.	
Lactobacillus, Bacillus cereus	4×10^7 cfu/g of diet	21 days	0.65 $\log_{10}$ cfu increased lactobacilli per gram of cecal digest	Li *et al.,* 2009
			0.75 $\log_{10}$ cfu increased *Bifidobacteria* per gram of cecal digest	
			0.69 $\log_{10}$ cfu decreased *Escherichia coli* per gram of cecal digest	
Lactobacillus, Bacillus subtilis, Saccharomyces cerevisiae, Aspergillus oryzae	Not specified	35 days	No effect on *Lactobacillus, Clostridium perfringens,* or *E. coli* in ileal digests	Woo *et al.,* 2006
Enterococcus faecium	2×10^7 cfu/g of diet	21 days	3.6 $\log_{10}$ cfu increased lactic acid bacteria per gram of ileal digest	Samli *et al.,* 2007
Bacillus subtilis	1×10^3 cfu/g of diet	21 days	1.6 $\log_{10}$ cfu decreased *Clostridium* spp.	Teo and Tan, 2007
			1.8 $\log_{10}$ decreased *E. coli*	
Bacillus subtilis	4.75×10^4 cfu/g of diet	18 weeks	2.9 $\log_{10}$ decreased pathogenic *E. coli* per gram of digest	Gebert *et al.,* 2007
			2.7 $\log_{10}$ decreased *Clostridium perfringens* type A per gram of digest	

[1] cfu, colony-forming units.

avian coccidiosis have been reported. In particular, dietary *Pediococcus* or *Lactobacillus* DFMs attenuated *Eimeria* challenge infections in broiler chickens resulting in increased body weight gain and decreased fecal shedding of infectious parasites compared with DFM-free controls (Dalloul *et al.,* 2005; Lee *et al.,* 2007a, b). Future metagenomics studies should focus on identifying the particular bacterial species positively linked with increased disease resistance or improved animal performance (Hattori and Taylor, 2009; Qu *et al.,* 2008).

Immunomodulation

DFMs influence the host immune system in multiple and diverse ways, including increased antibody production, up-regulation of cell-mediated immunity, promotion of epithelial barrier integrity, reduction of epithelial cell apoptosis, enhancement of dendritic cell-T cell interaction, improvement of T cell homing to mesenteric lymph nodes, and augmented Toll-like receptor signaling (Erickson and Hubbard, 2000; Corthesy *et al.,* 2007; Maldonado Galdeano *et al.,* 2007; Ng *et al.,* 2009). Many of these effects have been observed in the intestine of chickens experimentally challenged with enteric pathogens (Table 3). At the antibody level, birds that were fed DFMs and immunized with sheep red blood cells (SRBC)

Table 2. **Effects of DFMs on intestinal pathogen challenge studies in chickens**

DFMs used		Pathogen(s) challenged		Effect	Reference
Strain	Dose	Strain	Dose		
Lactobacillus	1×10^6 cfu[1]/ml drinking water	*Salmonella enterica* serovar Enteritis (SE)	1.8×10^4 cfu/chick	Significant SE reduction	Higgins *et al.,* 2008
Lactobacillus	1×10^{11} cfu/g of diet	*Salmonella enterica* serovar Typhimurium	1×10^7 cfu/chick	0.88 $\log_{10}$ decreased *Salmonella* per g of cecal digest	Revolledo *et al.,* 2009
				1.22 $\log_{10}$ decreased *Salmonella* cfu/g of liver	
				0.84 $\log_{10}$ decreased *Salmonella* cfu/g of spleen	
Lactobacillus	1×10^6 cfu/chick, single oral gavage	*Salmonella enterica* serovar Typhimurium	1×10^4 cfu/chick	ca. 3 $\log_{10}$ decreased *Salmonella* cfu/g of cecal digest	Haghighi *et al.,* 2008
Lactobacillus	1×10^5 cfu/g of diet	*Salmonella enterica* serotypes Typhimurium, Kentucky, Heidelberg	1×10^{10} cfu/chick	0.55 $\log_{10}$ decreased *Salmonella* cfu/g of lower intestinal tract	Grimes *et al.,* 2008
Lactobacillus	1×10^5 cfu/g of diet	*Eimeria acervulina*	2×10^4 oocysts/chick	13.3% decreased oocyst shedding	Dalloul *et al.,* 2005
Bacillus cereus var. *toyoi*	1×10^6 cfu/g of diet	*Salmonella enterica* serovar Enteritis	1×10^6 cfu/chick	7.0% increased BWG[1], 3.2% decreased FCR[1], 2.8% decreased mortality	Vilà *et al.,* 2009
Bacillus subtilis	1×10^3 cfu/g of diet	Pathogenic *E. coli* challenged at three time points	$0.8\text{-}4\times10^7$ cfu/chick	7.4% increased BWG, 7.7% decreased FCR, 6.0% decreased mortality	Teo and Tan, 2006
Pediococcus acidilactici, Saccharomyces boulardii	Not specified	*Eimeria tenella*	5×10^3 oocysts/chick	23.7% decreased oocyst shedding	Lee *et al.,* 2007b
Pediococcus acidilactici	Not specified	*Eimeria acervulina*	5×10^4 oocysts/chick	Increased BWG	Lee *et al.,* 2007a

[1] cfu, colony-forming units, BWG, body weight gain, FCR, feed conversion ratio.

as an experimental antigen produced higher anti-SRBC antibody titers compared with DFM-free controls (Haghighi *et al.,* 2005; Khaksefidi and Ghoorchi, 2006; Panda *et al.,* 2008). Specific antibody titers following immunization with Newcastle disease virus or *Eimeria* vaccines also were enhanced in DFM-fed chicks (Khaksefidi and Ghoorchi, 2006; Apata, 2008), illustrating the adjuvant role of DFMs with a practical relevance. Finally, the levels of pre-immune or natural antibodies to tetanus toxoid, *C. perfringens* alpha-toxin, and bovine serum albumin were increased in unimmunized DFM-fed chickens compared with DFM-free controls (Cetin *et al.,* 2005; Haghighi *et al.,* 2006).

At the cellular level, broiler chickens fed with a *Lactobacillus*-based DFM exhibited increased percentages of $CD3^+$, $CD4^+$, $CD8^+$, and $TCR2^+$ intestinal intraepithelial lymphocytes (IELs) compared with DFM-free controls (Dalloul *et al.,* 2003). Nousaium *et al.* (2008) observed that chickens infected with *Salmonella enterica* serovar Enteritis (SE) and fed with a *Lactobacillus* DFM displayed an increased percentage of $CD3^+$ lymphocytes, but reduced $CD4^+$ and $CD8^+$ cells, in the GIT compared with DFM-free controls. In addition, DFMs increased spontaneous as well as antigen-specific spleen lymphocyte proliferation in chickens, a surrogate marker of increased cellular immunity (Dalloul *et al.,* 2003; Koenen *et al.,* 2004; Lee *et al.,* 2007b; Nousaium *et al.,* 2008). Finally, Farnell *et al.* (2006) reported that

Table 3. **Effects of DFMs on immune responses in naïve and pathogen-challenged poultry**

Host	Route administered	DFM strain(s)	Main finding(s)	Reference
Naïve broilers	Feed	*Lactobacillus*	Increased $CD3^+$, $CD4^+$, $CD8^+$, and $TCR2^+$ intraepithelial lymphocytes	Dalloul *et al.,* 2003
Naïve broilers	Feed	*Lactobacillus*	Altered mRNA levels for IL-1β, IL-6, and IL-10	Chichlowski *et al.,* 2007b
Naïve broilers	Drinking water	*Bacillus subtilis, Lactobacillus*	Increased IgA levels in intestinal fluid and IgG-, IgM-, and IgA-forming cells	Yurong *et al.,* 2005
Naïve broilers	Oral gavage	*Lactobacillus*	Increased natural antibodies in serum and intestinal contents	Haghighi *et al.,* 2006
Naïve turkeys	Feed	*Lactobacillus*	Increased IgG and IgM levels	Cetin *et al.,* 2005
Naïve broilers	Oral gavage	*Bacillus subtilis*	Increased heterophil degranulation and oxidative burst	Farnell *et al.,* 2006
SRBC[1]-immunized broilers	Oral gavage	*Lactobacillus*	Increased anti-SRBC antibody titers	Haghighi *et al.,* 2005
SRBC-immunized poultry	Feed	*Bacillus*	Increased anti-SRBC antibody titers	Panda *et al.,* 2008; Khaksefidi and Ghoorchi, 2006
Eimeria-infected broilers	Feed	*Pediococcus acidilactici*	Increased anti-*Eimeria* antibody titers	Lee *et al.,* 2007a, b
Newcastle disease virus-vaccinated broilers	Feed	*Lactobacillus bulgaricus*	Increased anti-Newcastle disease virus antibody titers	Apata, 2008
TNP-KLH[1]-immunized broilers	Feed	*Lactobacillus paracasei*	Increased TNP-KLH-stimulated splenocyte proliferation	Koenen *et al.,* 2004
Salmonella-infected broilers	Oral gavage	*Lactobacillus acidophilus*	Increased $CD3^+$ lymphocytes and decreased $CD4^+$ and $CD8^+$ lymphocytes in the gastrointestinal tract	Nousaim *et al.,* 2008
Salmonella-infected broilers	Oral gavage	*Lactobacillus*	Increased phagocytic activity	Higgins *et al.,* 2007
Salmonella-infected chickens	Oral gavage	*Lactobacillus*	Decreased IL-12 and IFN-γ mRNA levels in cecal tonsils	Haghighi *et al.,* 2008
			No differences in IL-6 or IL-10 mRNA levels	
Chicken lymphoid cells	*In vitro* study	*Lactobacillus acidophilus*	Increased STAT2, STAT4, IL-18, MyD88, IFN-α, and IFN-γ mRNA levels	Brisbin *et al.,* 2008

[1]SRBC, sheep red blood cells, TNP-KLH, Trinitrophenyl-keyhole limpet hemocyanin.

DFMs increased heterophil degranulation and oxidative burst. Avian heterophils are the functional equivalents of mammalian neutrophils that comprise the second largest blood cell population and serve as critical components of innate immunity through their phagocytic and cytolytic actions mediated by reactive oxygen intermediates, proteolytic enzymes, and other microbicidal substances (Dar *et al.,* 2009).

On the basis of these collective studies, it is not surprising that the expression of chicken immune cytokines and chemokines have been shown to be drastically altered in response to a diet containing DFMs. Chichlowski *et al.* (2007b) demonstrated that chicks fed a diet supplemented with *Lactobacillus casei, L. acidophilus, Bifidobacterium*

Table 4. **Effect of *Bacillus subtilis* DFMs on immune parameters in broiler chickens**

Immune parameter	Control	15AP4	Bs27	AVICORR
IEL[2] subpopulations, %				
CD3	12.8	35.4*	47.9*	46.9*
CD4	0.7	0.9	12.9*	6.6*
CD8	13.7	22.6	56.8*	33.1*
TCR1	10.0	22.4*	23.7*	20.4*
TCR2	8.0	10.5	46.0*	34.9*
Cytokines[1]				
IL-1β	1.00	0.25	0.18†	1.10
IL-17α	1.00	17.99	2.06	294.16*
TNFSF15	1.00	1.09	1.21	2.41*
IFN-γ	1.00	0.52†	0.09†	0.48†
IL-2	1.00	0.16†	0.19†	1.38
IL-12	1.00	1.26	0.09	26.96*
IL-4	1.00	0.77	0.26	3.21*
IL-13	1.00	0.82	0.57	4.97*
NO^2 levels by IFN-γ-stimulated (μM)	6.82	6.30	7.23	13.89*
Phagocytosis (%)				
GFP[2]-labeled SE[2]	50.2	57.9*	55.3	53.3
Fluorescent beads per macrophage				
1-5 beads	28.3	31.6	47.2*	29.8
6-10 beads	8.8	10.7	17.2*	12.0
> 11 above beads	2.45	7.20*	15.85*	8.70*

[1] Values are expressed as relative expression levels compared with the control group.
* denotes significantly increased value compared with the control group ($P < 0.05$).
† denotes significantly decreased value compared with the control group ($P < 0.05$).
[2] IEL, intraepithelial lymphocyte, NO, nitric oxide, GFP, green fluorescent protein, SE, *Salmonella enterica* serovar Enteritidis.

thermophilum, and *Enterococcus faecium* for 21 days exhibited a decrease in intestinal mRNA levels of the pro-inflammatory cytokine IL-6, but an increase in the expression of the anti-inflammatory cytokine IL-10. On the other hand, another pro-inflammatory cytokine, IL-1β, was not affected by the DFM diet. The authors stated, however, that the significance of these alterations was not clear due to insufficient statistical power of the small sample size. In a recent DFM trial by Haghighi *et al.,* (2008), it was concluded that repression of IFN-γ and IL-12 expression levels in the chicken gut was associated with DFM-mediated reduction in intestinal colonization by *Salmonella enterica* serovar Typhimurium. By contrast, Fujiwara *et al.* (2009) observed no differences in the expression patterns of IFN-γ, IL-3, or IL-4 when birds were fed diets with or without *Bacillus subtilis*-fermented soybean. Finally, Brisbin *et al.* (2008) reported an up-regulation in the expression of the STAT2, STAT4, IL-18, MyD88, IFN-α, and IFN-γ genes in DFM-treated cecal tonsil cells using a chicken immune system microarray.

Effects of *Bacillus subtilis* DFMs on Chicken Immune Profiles

Given the reported effects of DFMs on chicken intestinal immunity, we conducted a series of experiments to assess the immunomodulatory properties of several *B. subtilis*-based probiotics. The bacterial strains included two purified cultures (15AP4 and Bs27) and one multi-component preparation (AVICORR, Danisco, WI, USA). All three DFMs exhibited growth inhibitory effects against avian pathogenic *E. coli* and *C. perfringens* type A. The immune parameters that were measured included intestinal IEL T cell subpopulations, cytokine mRNA levels in gut IELs, and macrophage activation (Table 4). IELs were chosen because they constitute the primary immune effector cells in the gut and play a critical role in eliciting protective immunity to enteric pathogens (Lillehoj *et al.,* 2004). Bs27- and AVICORR-supplemented diets increased the percentages of $CD3^+$, $CD4^+$, $CD8^+$, $TCR1^+$, and $TCR2^+$ IEL subpopulations compared with DFM-free controls. Increased levels of specific IEL T cell subsets by DFMs may contribute to increased host resistance to enteric pathogens which would otherwise cause clinical disease (Lillehoj and Trout, 1996). For the cytokine responses, IL-1β transcripts were decreased by Bs27, IFN-γ transcripts were lowered by all three DFMs, and IL-2 transcripts were decreased by 15AP4 and Bs27 compared with DFM-free controls. By contrast, IL-4, IL-12, IL-13, IL-17α, and TNFSF-15 transcripts were increased by AVICORR. In particular, the 294-fold increase in IL-17α transcripts in IELs from chickens fed AVICORR represented the greatest increase observed

among all cytokines in all treatment groups.

Macrophage activation in *B. subtilis*-based DFM-fed birds was assessed by measuring nitric oxide (NO) levels in cultures of IFN-γ-stimulated and by phagocytosis of fluorescent beads or green fluorescent protein (GFP)-labeled SE. NO levels were greater in AVICORR-fed birds compared with DFM-free controls (Table 4). In addition, the percentage of that engulfed GFP-SE was increased in 15AP4-fed chickens compared with the control group. DFM-fed birds also showed increased phagocytosis of fluorescent beads, with cells containing $\geq$ 11 beads being increased by 2.9–6.5-fold compared with controls. In concordance with these results, increased NO production by *Bifidobacterium* and *Lactobacillus* DFMs has been noted previously (Korhonen *et al.,* 2001; Kim *et al.,* 2007), and Higgins *et al.* (2007) reported enhanced phagocytic activity in *Salmonella*-infected, *Lactobacillus*-based DFMs-treated broiler chicks. However, the latter study failed to find any differences in the number of macrophages residing in the ileum or ceca of untreated or DFM-treated birds, suggesting an effect of DFMs on macrophage function rather than hyperplasia.

Conclusion and Future Directions

Current evidence indicates that DFMs impact the health and productivity in poultry through balancing of the intestinal microfloral population and by modulating gut immunity. Dietary DFMs inhibit enteric pathogens by direct interaction and indirectly by promoting the normal microflora to competitively exclude pathogens. Additionally, DFMs modulate humoral and cellular immune responses to enhance protective immunity. It is, however, necessary to further define the detailed molecular and cellular mechanisms that govern the multiple interactions between the intestinal microflora, pathogenic bacteria, and the host immune system before the full potential of DFMs can be applied to food animal production. Future studies should include multidisciplinary approaches to characterize the effects of DFMs on the chicken immune system at the genomic and molecular levels (for example by the use of high-throughput gene expression profiling), to study the immunoregulatory effects of DFMs on T cell subpopulations and their functions (such as Th1/Th2 balance or the development of IL-17-producing T regulatory cells), and to identify the bacterial species most effective in promoting disease resistance and/or growth performance through metagenomic analysis.

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