

◀Review▶

## 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 modulation 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).

### 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

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( $n=275$ , 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 *Escherichia 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, colibacillosis, 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
<i>Lactobacillus</i>	$3 \times 10^5$ cfu/g of diet	21 days	Less segmented filamentous-like bacteria (SFB) in ileum  Dense bacterial population on cecal surface	Chichlowski <i>et al.</i> , 2007a
<i>Lactobacillus</i>	Not specified	49 days	12% decreased prevalence of <i>Campylobacter jejuni</i>	Willis and Reid, 2008
<i>Lactobacillus</i>	$2 \times 10^6$ cfu <sup>1</sup> /g of diet	42 days	ca. $1.0 \log_{10}$ cfu increased <i>Bifidobacterium</i> spp., <i>Lactobacillus</i> spp., and Gram positive cocci per gram of cecal digest  No effect on total aerobes, coliforms, total anaerobes, or <i>Bacteroides</i> spp.	Mountzouris <i>et al.</i> , 2007
<i>Lactobacillus</i> , <i>Bacillus cereus</i>	$4 \times 10^7$ cfu/g of diet	21 days	$0.65 \log_{10}$ cfu increased lactobacilli per gram of cecal digest  $0.75 \log_{10}$ cfu increased <i>Bifidobacteria</i> per gram of cecal digest  $0.69 \log_{10}$ cfu decreased <i>Escherichia coli</i> per gram of cecal digest	Li <i>et al.</i> , 2009
<i>Lactobacillus</i> , <i>Bacillus subtilis</i> , <i>Saccharomyces cerevisiae</i> , <i>Aspergillus oryzae</i>	Not specified	35 days	No effect on <i>Lactobacillus</i> , <i>Clostridium perfringens</i> , or <i>E. coli</i> in ileal digests	Woo <i>et al.</i> , 2006
<i>Enterococcus faecium</i>	$2 \times 10^7$ cfu/g of diet	21 days	$3.6 \log_{10}$ cfu increased lactic acid bacteria per gram of ileal digest	Samli <i>et al.</i> , 2007
<i>Bacillus subtilis</i>	$1 \times 10^3$ cfu/g of diet	21 days	$1.6 \log_{10}$ cfu decreased <i>Clostridium</i> spp.  $1.8 \log_{10}$ decreased <i>E. coli</i>	Teo and Tan, 2007
<i>Bacillus subtilis</i>	$4.75 \times 10^4$ cfu/g of diet	18 weeks	$2.9 \log_{10}$ decreased pathogenic <i>E. coli</i> per gram of digest  $2.7 \log_{10}$ decreased <i>Clostridium perfringens</i> type A per gram of digest	Gebert <i>et al.</i> , 2007

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

<sup>1</sup> 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<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, and TCR2<sup>+</sup> 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 Enteritidis (SE) and fed with a *Lactobacillus* DFM displayed an increased percentage of CD3<sup>+</sup> lymphocytes, but reduced CD4<sup>+</sup> and CD8<sup>+</sup> 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	<i>Lactobacillus</i>	Increased CD3 <sup>+</sup> , CD4 <sup>+</sup> , CD8 <sup>+</sup> , and TCR2 <sup>+</sup> intraepithelial lymphocytes	Dalloul <i>et al.</i> , 2003
Naïve broilers	Feed	<i>Lactobacillus</i>	Altered mRNA levels for IL-1 $\beta$ , IL-6, and IL-10	Chichlowski <i>et al.</i> , 2007b
Naïve broilers	Drinking water	<i>Bacillus subtilis</i> , <i>Lactobacillus</i>	Increased IgA levels in intestinal fluid and IgG-, IgM-, and IgA-forming cells	Yurong <i>et al.</i> , 2005
Naïve broilers	Oral gavage	<i>Lactobacillus</i>	Increased natural antibodies in serum and intestinal contents	Haghighi <i>et al.</i> , 2006
Naïve turkeys	Feed	<i>Lactobacillus</i>	Increased IgG and IgM levels	Cetin <i>et al.</i> , 2005
Naïve broilers	Oral gavage	<i>Bacillus subtilis</i>	Increased heterophil degranulation and oxidative burst	Farnell <i>et al.</i> , 2006
SRBC <sup>1</sup> -immunized broilers	Oral gavage	<i>Lactobacillus</i>	Increased anti-SRBC antibody titers	Haghighi <i>et al.</i> , 2005
SRBC-immunized poultry	Feed	<i>Bacillus</i>	Increased anti-SRBC antibody titers	Panda <i>et al.</i> , 2008; Khaksefidi and Ghoorchi, 2006
<i>Eimeria</i> -infected broilers	Feed	<i>Pediococcus acidilactici</i>	Increased anti- <i>Eimeria</i> antibody titers	Lee <i>et al.</i> , 2007a, b
Newcastle disease virus-vaccinated broilers	Feed	<i>Lactobacillus bulgaricus</i>	Increased anti-Newcastle disease virus antibody titers	Apata, 2008
TNP-KLH <sup>1</sup> -immunized broilers	Feed	<i>Lactobacillus paracasei</i>	Increased TNP-KLH-stimulated splenocyte proliferation	Koenen <i>et al.</i> , 2004
<i>Salmonella</i> -infected broilers	Oral gavage	<i>Lactobacillus acidophilus</i>	Increased CD3 <sup>+</sup> lymphocytes and decreased CD4 <sup>+</sup> and CD8 <sup>+</sup> lymphocytes in the gastrointestinal tract	Nousaim <i>et al.</i> , 2008
<i>Salmonella</i> -infected broilers	Oral gavage	<i>Lactobacillus</i>	Increased phagocytic activity	Higgins <i>et al.</i> , 2007
<i>Salmonella</i> -infected chickens	Oral gavage	<i>Lactobacillus</i>	Decreased IL-12 and IFN- $\gamma$ mRNA levels in cecal tonsils  No differences in IL-6 or IL-10 mRNA levels	Haghighi <i>et al.</i> , 2008
Chicken lymphoid cells	<i>In vitro</i> study	<i>Lactobacillus acidophilus</i>	Increased STAT2, STAT4, IL-18, MyD88, IFN- $\alpha$ , and IFN- $\gamma$ mRNA levels	Brisbin <i>et al.</i> , 2008

<sup>1</sup>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 <sup>2</sup> 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 <sup>1</sup>				
IL-1 $\beta$	1.00	0.25	0.18†	1.10
IL-17 $\alpha$	1.00	17.99	2.06	294.16*
TNFSF15	1.00	1.09	1.21	2.41*
IFN- $\gamma$	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 <sup>2</sup> levels by IFN- $\gamma$ -stimulated ( $\mu$ M)	6.82	6.30	7.23	13.89*
Phagocytosis (%)				
GFP <sup>2</sup> -labeled SE <sup>2</sup>	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*

<sup>1</sup> 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 ( $P < 0.05$ ).

<sup>2</sup> 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-1 $\beta$ , 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 Haghghi *et al.*, (2008), it was concluded that repression of IFN- $\gamma$  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- $\gamma$ , 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- $\alpha$ , and IFN- $\gamma$  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<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, TCR1<sup>+</sup>, and TCR2<sup>+</sup> 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-1 $\beta$  transcripts were decreased by Bs27, IFN- $\gamma$  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-17 $\alpha$ , and TNFSF-15 transcripts were increased by AVICORR. In particular, the 294-fold increase in IL-17 $\alpha$  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- $\gamma$ -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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### References

- Amit-Romach E, Sklan D and Uni Z. Microflora ecology of the chicken intestine using 16S ribosomal DNA primers. *Poultry Science*, 83: 1093–1098. 2004.
- Apajalahti J, Kettunen A and Graham H. Characteristics of the gastrointestinal microbial communities, with special reference to the chicken. *World's Poultry Science Journal*, 60: 223–232. 2004.
- Apata DF. Growth performance, nutrient digestibility and immune response of broiler chicks fed diets supplemented with a culture of *Lactobacillus bulgaricus*. *Journal of the Science of Food and Agriculture*, 88: 1253–1258. 2008.
- Beal RK, Powers C, Davison TF and Smith AL. Immunological development of the avian gut. In: *Avian Gut Function in Health and Disease* (Perry GC ed.). Vol.28. pp. 85–103. CABI Publishing. Oxford. 2006.
- Brisbin JT, Zhou H, Gong J, Sabour P, Akbari MR, Haghghi HR, Yu H, Clarke A, Sarson AJ and Sharif S. Gene expression profiling of chicken lymphoid cells after treatment with *Lactobacillus acidophilus* cellular components. *Developmental and Comparative Immunology*, 32: 563–574. 2008.
- Callaway TR, Edrington TS, Anderson RC, Harvey RB, Genovese KJ, Kennedy CN, Venn DW and Nisbet DJ. Probiotics, prebiotics and competitive exclusion for prophylaxis against bacterial disease. *Animal Health Research Reviews*, 9: 217–225. 2008.
- Cebra JJ. Influences of microbiota on intestinal immune system development. *American Journal of Clinical Nutrition*, 69: 1046S–1051S. 1999.
- Cetin N, Guclu BK and Cetin E. The effects of probiotic and mannanoligosaccharide on some haematological and immunological parameters in turkeys. *Journal of Veterinary Medicine A*, 52: 263–267. 2005.
- Chichlowski M, Croom WJ, Edens FW, McBride BW, Qiu R, Chiang CC, Daniel LR, Havenstein GB and Koci MD. Microarchitecture and spatial relationship between bacterial and ileal, cecal, and colonic epithelium in chicks fed a direct-fed microbial, PrimaLac, and salinomycin. *Poultry Science*, 86: 1121–1132. 2007a.
- Chichlowski M, Croom J, McBride BW, Daniel L, Davis G and Koci MD. Direct-fed microbial PrimaLac and salinomycin modulate whole-body and intestinal oxygen consumption and intestinal mucosal cytokine production in the broiler chick. *Poultry Science*, 86: 1100–1106. 2007b.
- Choct M. Managing gut health through nutrition. *British Poultry Science*, 50: 9–15. 2009.
- Corthesy B, Gaskins HR and Mercenier A. Cross-talk between probiotic bacteria and the host immune system. *Journal of Nutrition*, 137: 781S–790S. 2007.
- Dahiya JP, Wilkie DC, van Kessel AG and Drew MD. Potential strategies for controlling necrotic enteritis in broiler chickens in post-antibiotic era. *Animal Feed Science and Technology*, 129: 60–88. 2006.
- Dalloul RA, Lillehoj HS, Shellem TA and Doerr JA. Enhanced mucosal immunity against *Eimeria acervulina* in broilers fed a *Lactobacillus*-based probiotic. *Poultry Science*, 82: 62–66. 2003.
- Dalloul RA, Lillehoj HS, Tamim NM, Shellem TA and Doerr JA. Induction of local protective immunity to *Eimeria acervulina* by a *Lactobacillus*-based probiotic. *Comparative Immunology, Microbiology and Infectious Diseases*, 28:

- 351–361. 2005.
- Dar A, Allan B, Gomis S, Potter A and Mutwiri G. Immunotherapeutic potential of CpG oligonucleotides in chickens. *Journal of Poultry Science*, 46: 69–80. 2009.
- Donoghue AM, Farnell MB, Cole K and Donoghue DJ. Mechanisms of pathogen control in the avian gastrointestinal tract. In: *Avian Gut Function in Health and Disease* (Perry GC ed.). Vol.28. pp. 138–155. CABI Publishing. Oxford. 2006.
- Erickson K and Hubbard NE. Probiotic immunomodulation in health and disease. *Journal of Nutrition*, 130: 403S–409S. 2000.
- FAO/WHO. Guidelines for the Evaluation of Probiotics in Food. Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food. Ontario, Canada. 2002.
- Farnell MB, Donoghue AM, Solis de los Santos F, Blore PJ, Hargis BM, Tellez G and Donoghue DJ. Upregulation of oxidative burst and degranulation in chicken heterophils stimulated with probiotic bacteria. *Poultry Science*, 85: 1900–1906. 2006.
- Fuentes S, Egert M, Jimenez-Valera M, Monteoliva-Sanchez M, Ruiz-Bravo A and Smidt H. A strain of *Lactobacillus plantarum* affects segmented filamentous bacteria in the intestine of immunosuppressed mice. *FEMS Microbiology Ecology*, 63: 65–72. 2008.
- Fujiwara K, Yamazaki M, Abe H, Nakashima K, Yakabe Y, Otsuka M, Ohbayashi Y, Kato Y, Namai K, Toyoda A, Miyaguchi Y and Nakamura Y. Effect of *Bacillus subtilis* var. *natto* fermented soybean on growth performance, microbial activity in the caeca and cytokine gene expression of domestic meat type chickens. *Journal of Poultry Science*, 46: 116–122. 2009.
- Gabriel I, Lessire M, Mallet S and Guillot JF. Microflora of the digestive tract: critical factors and consequences for poultry. *World's Poultry Science Journal*, 62: 499–511. 2006.
- Gebert S, Kromm C and Rehberger T. Effect of a *Bacillus*-based direct-fed microbial on turkey poult performance and changes within the gastrointestinal microflora. *Poultry Science*, 86(suppl. 1): 249(abstract). 2007.
- Grimes JL, Rahimi S, Oviedo E, Sheldon BW and Santos FBO. Effects of a direct-fed microbial (Primalac) on turkey poult performance and susceptibility to oral *Salmonella* challenge. *Poultry Science*, 87: 1464–1470. 2008.
- Haghighi HR, Gong J, Gyles CL, Anthony Hayes M, Sanei B, Parvizi P, Gisavi H, Chambers JR and Sharif S. Modulation of antibody-mediated immune response by probiotics in chickens. *Clinical and Diagnostic Laboratory Immunology*, 12: 1387–1392. 2005.
- Haghighi HR, Gong J, Gyles CL, Anthony Hayes M, Zhou H, Sanei B, Chambers JR and Sharif S. Probiotics stimulate production of natural antibodies in chickens. *Clinical and Vaccine Immunology*, 13: 975–980. 2006.
- Haghighi HR, Abdul-Careem MF, Dara RA, Chambers JR and Sharif S. Cytokine gene expression in chicken cecal tonsils following treatment with probiotics and *salmonella* infection. *Veterinary Microbiology*, 126: 225–233. 2008.
- Hariharan H, Murphy GA and Kempf I. *Campylobacter jejuni*: Public health hazards and potential control methods in poultry: a review. *Veterinary Medicine — Czech*, 49: 441–446. 2004.
- Hattori M and Taylor TD. The human intestinal microbiome: A new frontier of human biology. *DNA Research*, 16: 1–12. 2009.
- Higgins SE, Erf GF, Higgins JP, Henderson SN, Wolfenden AD, Gaona-Ramirez G and Hargis BM. Effect of probiotic treatment in broiler chicks on intestinal macrophage numbers and phagocytosis of *Salmonella* Enteritidis by abdominal exudate cells. *Poultry Science*, 86: 2315–2312. 2007.
- Higgins SE, Higgins JP, Wolfenden AD, Henderson SN, Torres-Rodriguez A, Tellez G and Hargis B. Evaluation of a *Lactobacillus*-based probiotic culture for the reduction of *Salmonella* Enteritidis in neonatal broiler chicks. *Poultry Science*, 87: 27–31. 2008.
- Kabir SML. The role of probiotics in the poultry industry. *International Journal of Molecular Sciences*, 10: 3531–3546. 2009.
- Khaksefidi A and Ghoorchi T. Effect of probiotic on performance and immunocompetence in broiler chicks. *Journal of Poultry Science*, 43: 296–300. 2006.
- Kim DW, Cho SB, Lee HJ, Chung WT, Kim KH, Hwangbo J, Nam IS, Cho YI, Yang MP and Chung IB. Comparison of cytokine and nitric oxide induction in murine macrophages between whole cell and enzymatically digested *Bifidobacterium* sp. obtained from monogastric animals. *Journal of Microbiology*, 45: 305–310. 2007.
- Koenen ME, Boonstra-Blom AG and Jeurissen SHM. Immunological differences between layer- and broiler-type chickens. *Veterinary Immunology and Immunopathology*, 89: 47–56. 2002.
- Koenen ME, Kramer J, van der Hulst R, Heres L, Jeurissen SHM and Boersma WJA. Immunomodulation by probiotic lactobacilli in layer- and meat-type chickens. *British Poultry Science*, 45: 355–366. 2004.
- Korhonen R, Korpela R, Saxelin M, Mäki M, Kankaanranta H and Moilanen E. Induction of nitric oxide synthesis by probiotic *Lactobacillus rhamnosus* GG in J774 macrophages and human T84 intestinal epithelial cells. *Inflammation*, 25: 223–232. 2001.
- Lee KW, Lee SK and Lee BD. *Aspergillus oryzae* as probiotic in poultry — A review. *International Journal of Poultry Science*, 5: 1–3. 2006.
- Lee SH, Lillehoj HS, Dalloul RA, Park DW, Hong YH and Lin JJ. Influence of *Pediococcus*-based probiotic on coccidiosis in broiler chickens. *Poultry Science*, 86: 63–66. 2007a.
- Lee SH, Lillehoj HS, Park DW, Hong YH and Lin JJ. Effects of *Pediococcus*- and *Saccharomyces*-based probiotic (MitoMax<sup>®</sup>) on coccidiosis in broiler chickens. *Comparative Immunology, Microbiology and Infectious Diseases*, 30: 261–267. 2007b.
- Li SP, Zhao XJ and Wang JY. Synergy of Astragalus polysaccharides and probiotics (*Lactobacillus* and *Bacillus cereus*) on immunity and intestinal microbiota in chicks. *Poultry Science*, 88: 519–525. 2009.
- Lillehoj HS and Trout JM. Avian gut-associated lymphoid tissues and intestinal immune responses to *Eimeria* parasites. *Clinical Microbiology Reviews*, 9: 349–360. 1996.
- Lillehoj HS, Min W and Dalloul RA. Recent progress on the cytokine regulation of intestinal immune responses to *Eimeria*. *Poultry Science*, 83: 611–623. 2004.
- Lowenthal JW, Connick TE, McWaters PG and York JJ. Development of T cell immune responsiveness in the chicken. *Immunology and Cell Biology*, 72: 115–122. 1994.
- Maldonado Galdeano C, de Moreno de LeBlanc A, Vinderola G, Bibas Bonet ME and Perdigon G. Proposed model: mecha-



- nisms of immunomodulation induced by probiotic bacteria. *Clinical and Vaccine Immunology*, 14: 485-492. 2007.
- Mountzouris KC, Tsirtsikos P, Kalamara E, Nitsch S, Schatzmayr G and Fegeros K. Evaluation of the efficacy of a probiotic containing *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, and *Pediococcus* strains in promoting broiler performance and modulating cecal microflora composition and metabolic activities. *Poultry Science*, 86: 309-317. 2007.
- Ng SC, Hart AL, Kamm MA, Stagg AJ and Knight SC. Mechanisms of action of probiotics: recent advances. *Inflammatory Bowel Diseases*, 15: 300-310. 2009.
- Nousaim JC, Andreatti Filho L, Lima ET, Okamoto AS, Amorim RL and Torres Nete R. Detection of T lymphocytes in intestine of broiler chicks treated with *Lactobacillus* spp. and challenged with *Salmonella enterica* serovar Enteritidis. *Poultry Science*, 87: 927-933. 2008.
- Nurmi E and Rantala M. New aspects of *Salmonella* infection in broiler production. *Nature*, 241: 210-211. 1973.
- O'Hara AM and Shanahan F. The gut flora as a forgotten organ. *EMBO Reports*, 7: 688-693. 2006.
- Panda AK, Rama Rao SS, Raju MVLN and Sharma SS. Effect of probiotic (*Lactobacillus sporogenes*) feeding on egg production and quality, yolk cholesterol and humoral immune response of White Leghorn layer breeders. *Journal of the Science of Food and Agriculture*, 88: 43-47. 2008.
- Patterson JA and Burkholder KM. Application of prebiotics and probiotics in poultry production. *Poultry Science*, 82: 627-631. 2003.
- Qu A, Brulc JM, Wilson MK, Law BF, Theoret JR, Joens LA, Kondel ME, Angly F, Dinsdale EA, Edwards RA, Nelson KE and White BA. Comparative metagenomics reveals host specific Metavirulomes and horizontal gene transfer elements in the chicken cecum microbiome. *PLoS ONE*, 3: e 2945. 2008.
- Rehberger TG and Jordan-Parrott DS. Methods and composition for reducing *E. coli* disease and enhancing performance. US Pat. No. US 2005/0255092 A1. 2005.
- Reid G and Friendship R. Alternative to antibiotic use: probiotics for the gut. *Animal Biotechnology*, 13: 97-112. 2002.
- Revolledo L, Ferreira CSA and Ferreira AJP. Prevention of *Salmonella* Typhimurium colonization and organ invasion by combination treatment in broiler chicks. *Poultry Science*, 88: 783-743. 2009.
- Rhee K, Sethupathi P, Driks A, Lanning DK and Knight KL. Role of commensal bacteria in development of gut-associated lymphoid tissues and preimmune antibody repertoire. *Journal of Immunology*, 172: 1118-1124. 2004.
- Salminen S, Bouley C, Boutron-Ruault MC, Cummings JH, Franck A, Gibson GR, Isolauri E, Moreau MC, Roberfroid M and Rowland I. Functional food science and gastrointestinal physiology and function. *British Journal of Nutrition*, 80: S147-S171. 1998.
- Samli HE, Senkoylu N, Koc F, Kanter M and Agma A. Effects of *Enterococcus faecium* and dried whey on broiler performance, gut histomorphology and intestinal microbiota. *Archives of Animal Nutrition*, 61: 42-49. 2007.
- Shima T, Fukushima K, Setoyama H, Imaoka A, Matsumoto S, Hara T, Suda K and Umesaki Y. Differential effects of two probiotic strains with different bacteriological properties on intestinal gene expression, with special reference to indigenous bacteria. *FEMS Immunology and Medical Microbiology*, 52: 69-77. 2008.
- Smith JA and Helm JD. Report of the committee on transmissible diseases of poultry and other avian species. 2008. United States Animal Health Association. Available Source: <http://www.usaha.org/committees/reports/2008/report-pad-2008.pdf>.
- Teo AY and Tan HM. Effect of *Bacillus subtilis* PB6 (CloSTAT) on broilers infected with a pathogenic strain of *Escherichia coli*. *Journal of Applied Poultry Research*, 15: 229-235. 2006.
- Teo AY and Tan HM. Evaluation of the performance and intestinal gut microflora of broilers fed on corn-soy diets supplemented with *Bacillus subtilis* PB6 (CloSTAT). *Journal of Applied Poultry Research*, 16: 296-303. 2007.
- Torok VA, Ophel-Keller K, Loo M and Hughes RJ. Application of methods for identifying broiler chicken gut bacterial species linked with increased energy metabolism. *Applied and Environmental Microbiology*, 74: 783-791. 2008.
- Vilà B, Fontgibell A, Badiola I, Esteve-Garcia E, Jiménez G, Castillo M and Brufau J. Reduction of *Salmonella enterica* var. Enteritidis colonization and invasion by *Bacillus cereus* var. *toyoi* inclusion in poultry feeds. *Poultry Science*, 88: 975-979. 2009.
- Web of Science. ISI Web of Knowledge. <http://www.isiknowledge.com/Accessed 15 September, 2009>.
- Willis WL and Reid L. Investigating effects of dietary probiotic regimens on broiler chicken production and *Campylobacter jejuni* presence. *Poultry Science*, 87: 606-611. 2008.
- Woo KC, Jung BY, Lee MK and Paik IK. Effects of supplementary Safmannan<sup>®</sup> (beta-glucan and MOS) and World-Labs<sup>®</sup> (multiple probiotics) on the performance, nutrient availability, small intestinal microflora and immune response in broiler chicks. *Korean Journal of Poultry Science*, 33: 151-158. 2006.
- Yang Y, Iji PA and Choct M. Dietary modulation of gut microflora in broiler chickens: a review of the role of six kinds of alternatives to in-feed antibiotics. *World's Poultry Science Journal*, 65: 97-114. 2009.
- Yurong Y, Ruiping S, Shimin Z and Yibao J. Effect of probiotics on intestinal mucosal immunity and ultrastructure of cecal tonsils of chickens. *Archives of Animal Nutrition*, 59: 237-246. 2005.
- Zhang AW, Lee BD, Lee KW, Song KB, Ahn GH and Lee CH. Effects of yeast (*Saccharomyces cerevisiae*) cell components on growth performance, meat quality, and ileal mucosa development of broiler chicks. *Poultry Science*, 84: 1015-1021. 2005.