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Direct fed microbial supplementation repartitions host energy to the immune system[1]

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ABSTRACT: Direct fed microbials and probiotics are used to promote health in livestock and poultry; however, their mechanism of action is still poorly understood. We previously reported that direct fed microbial supplementation in young broilers reduced ileal respiration without changing whole-body energy expenditure. The current studies were conducted to further investigate the effects of a direct fed microbial on energy metabolism in different tissues of broilers. One hundred ninety-two 1-d-old broiler chicks (16 chicks/pen) were randomly assigned to 2 dietary groups: standard control starter diet (CSD) and CSD plus direct fed microbial (DFMD; 0.3%) with 6 pens/treatment. Body weight, feed consumption, whole-body energy expenditure, organ mass, tissue respiration rates, and peripheral blood mononuclear cell (PBMC) ATP concentrations were measured to estimate changes in energy metabolism. No differences in whole body energy expenditure or BW gain were observed; however, decreased ileal O_2 respiration ($P < 0.05$) was measured in DFMD fed broilers. In contrast, the respiration rate of the thymus in those broilers was increased ($P < 0.05$). The PBMC from DFMD fed broilers had increased ATP concentrations and exhibited increased ATP turnover ($P < 0.01$). To determine if the increased energy consumption by PBMC corresponded with an altered immune response, broilers were immunized with sheep red blood cells (SRBC) and assayed for differences in their humoral response. The DFMD-fed broilers had a faster rate of antigen specific IgG production ($P < 0.05$) and an increase in total IgA ($P < 0.05$). Collectively, these data indicate that supplementation with the direct fed microbial used in this study resulted in energy re-partitioning to the immune system and an increase in antibody production independent of changes in whole body metabolism or growth performance.

Key words: broiler, direct fed microbials, energy metabolism, gene expression, probiotics, systemic immune function

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INTRODUCTION

The microorganisms of the intestinal microbiota play an important role in the health, growth, and development of the host (O'Hara and Shanahan, 2006). Those bacteria have been reported to influence the intestinal barrier, immune function, and digestion (Noverr and Huffnagle, 2005; Haghighi et al., 2006; Wen et al., 2008; Turnbaugh and Gordon, 2009; Velagapudi et al., 2010). Direct fed microbials and probiotics have been used in animal and human nutrition to exploit these effects and promote health for over a century (Metchnikoff and Chalmers, 1910; Edens, 2003). In spite of widespread use, their mechanism of action is not fully understood. However, 2 hypotheses are frequently proposed: 1) direct fed microbials aid in

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the control of pathogens (Prescott et al., 2005; Galdeano and Perdigon, 2006) or 2) promote digestion (Champ et al., 1981) and overall gut function (Madsen et al., 2001; Chichlowski et al., 2007a). A more detailed understanding of the mechanisms of microbiota communication with the host will lead to novel disease prevention and optimum production strategies.

Previous studies have investigated the effects of direct fed microbial supplementation on animal and intestinal O_2 consumption using the rapidly growing broiler chick model. No differences in growth, feed conversion, or whole animal O_2 consumption were noted; however, a decrease in small intestinal O_2 consumption and a transient increase in gut length was observed (Chichlowski et al., 2007b). Given the fact that the small intestines from animals fed supplemented diets consumed less O_2 than controls yet their whole body metabolic rates were similar led us to hypothesize that direct fed microbial supplementation results in repartitioning of energy among the body tissues (Mitchell, 1962; Obst and Diamond, 1992; Cant et al., 1996; Fan et al., 1997). The current study was conducted to further characterize the effects of direct fed microbials on tissue O_2 consumption, repartitioning of energy to the immune system, and subsequent immune function.

MATERIALS AND METHODS

Broiler chicks were housed, maintained, and euthanized under an approved protocol from the Institutional Animal Care and Use Committee at North Carolina State University.

Broiler Chicks and Diets

One hundred ninety-two, 1-d-old male broiler chicks were randomly assigned to 1 of 2 dietary treatments with 6 pens per treatment and 16 chicks per pen. One treatment group was fed a standard control starter diet (**CSD**, Table 1). The second treatment group was fed a diet supplemented with the direct fed microbial (Primalac; Star Labs Inc., Clarksdale, MO). This commercially available direct fed microbial has been previously reported to improve growth, performance, and disease response (Maxwell et al., 1983; Davis and Anderson, 2002; Dalloul et al., 2003; Chichlowski et al., 2007a). This direct fed microbial is supplied as dry pre-mix containing 3×10^5 cfu/g *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifido bacterium bifidium*, and *Enterococcus faecium* (Star Labs Inc.). The direct fed microbial diet (**DFMD**) was prepared fresh before each independent experiment by adding the pre-mix to a CSD basal diet (0.3%) as previously described (Chichlowski et al., 2007a,b). Samples of the premix (data not shown), DFMD, and CSD were collected to confirm the presence of viable direct fed microbial genera (Table 1) before each experiment.

Table 1. Experimental diet[1]

Item	CSD	DFMD
Ingredient, %		
Corn, 8.5% CP	51.66	51.66
Soybean meal	37.96	37.96
Poultry fat	6.00	6.00
Dicalcium phosphate	1.02	1.02
Limestone	2.09	2.09
Salt	0.50	0.50
Choline chloride 60	0.20	0.20
Mineral premix[2]	0.20	0.20
DL-methionine	0.164	0.164
Selenium premix (0.02% Se)	0.10	0.10
L-Threonine	0.056	0.056
Vitamin premix[3]	0.05	0.05
Microbial species		
Lactobacilli[4]	7.10 ± 0.02	7.91 ± 0.02
Bifidobacteria[4]	7.07 ± 0.14	7.89 ± 0.03
Enterococcus [4]	7.20 ± 0.03	7.45 ± 0.17
Enterococcus faecium[5]	ND[6]	86.5 ± 17.7

[1]CSD = standard control starter diet, and DFMD = direct fed microbial diet. Analytical results (%): protein, 23.8; Ca, 0.96; and P, 0.46%.

[2]PX NCSU BR Mineral (North Carolina State University, Raleigh, NC), containing (per kilogram of diet): manganese ($MnSO_4$), 120 mg; zinc ($ZnSO_4$), 120 mg; iron ($FeSO_4$), 80 mg; copper ($CuSO_4$), 10 mg; iodine ($Ca(IO_3)_2$), 2.5 mg; and cobalt ($CoSO_4$), 1 mg.

[3]PX NCSU BR Vitamin (North Carolina State University, Raleigh, NC), containing (per kilogram of diet): vitamin A, 5,291 IU; vitamin D_3, 1,587 IU; vitamin E, 26.5 mg; vitamin B_{12}, 16 μg; riboflavin, 5.3 mg; niacin, 44.1 mg; d-pantothenic acid, 8.8 mg; vitamin K_3, 1.6 mg; folic acid, 0.9 mg; thiamine, 1.6 mg; and d-biotin, 100.6 μg.

[4]$Log_{(10)}$ cfu/g.

[5]*Enterococcus faecium* genomes/μg of feed DNA as determined by quantitative PCR.

[6]ND = none detected.

Twelve broilers from each diet group were immunized intravenously with 7% sheep red blood cells (**SRBC**) in PBS (pH 7.4, packed cell vol/vol) at d 7 and re-immunized at d 14 and 21. Serum samples were collected at 10, 14, 16, 21, and 28 d of age as previously described (Aslam et al., 1998). Broilers in both groups were fed their respective diets, ad libitum, for the duration of the experiment as previously described (Chichlowski et al., 2007b).

Bacteriology

The numbers of viable *Lactobacillus*, *Bifidobacteria*, and *Enterococcus* species were determined as previously described (Tharmaraj and Shah, 2003). Samples of premix and mixed diets were incubated at room temp for 18 h (1 g/100 mL in PBS, pH 7.8) with 0.1% Tween-20 before enumeration. Samples were then subjected to serial 10-fold dilutions, and 500 μL of each dilution was plated onto media (*Lactobacillus* = MRS agar; *Bifidobacteria* = BS-LV agar, and *Enterococcus* = KF *Streptococcus* agar). The KF *Streptococcus* agar plates were incubated

aerobically, whereas MRS agar and BS-LV agar were incubated anaerobically (anaerobic jars, GasPak System; Becton Dickinson, Franklin Lakes, NJ) for 48 h at 42°C. Colony counts were obtained and expressed as a $\log_{10}$ of the cfu/g sample. The specific organisms in the direct fed microbial used in these studies do not contain unique antibiotic resistance markers and, therefore, cannot be differentiated from other species of *Lactobacillus*, *Bifidobacteria*, or *Enterococcus* that may be present in a given sample using classical culture methodology.

Detection of direct fed microbial species in the 2 treatment diets was accomplished by quantitative PCR, using *Enterococcus faecium* as an indicator organism. Total DNA was isolated from 200 mg of CSD or DFMD feed (QIAamp DNA Stool Mini Kit; Qiagen, Valencia, CA). The amount of *Enterococcus faecium* present was then determined by real-time PCR (TaqMan Gene Expression Master Mix and StepOnePlus Real-Time PCR System; Lifetechnologies, Grand Island, NY) and compared with a standard curve (*Enterococcus faecium* Genomes genesig standard kit; PrimerDesign Ltd, Rownhams, Southampton, UK). The amount of *Enterococcus faecium* was quantitated by averaging the cycle threshold (**Ct**) of triplicate wells of each diet DNA and plotting against a standard curve (StepOne Software v2.1; Life Technologies).

Whole Body O_2 Consumption

Whole body O_2 consumption was estimated as previously described (Chichlowski et al., 2007b). Six non-immunized broilers per treatment were individually measured at 3, 8, 15, 22, and 28 d of age using an O_2-ECO system (Columbus Instruments International, Columbus, OH). Each broiler was allowed a 15 min adjustment period in the chamber with an air flow rate of 2.5, 3, 3, 3.5, or 3.5 L/min at 3, 8, 15, 22, or 28 d of age, respectively. Oxygen consumption and CO_2 expiration were measured during 3 consecutive 60-s periods over a total of 12 min. The BW of each bird was measured immediately after the measurement of gas exchange and mean O_2 consumption and CO_2 expiration were expressed as μmol/min per gram of BW.

Tissue O_2 Consumption

Six non-immunized broilers/treatment (1 broiler from each pen) were euthanized at 8, 15, and 22 d of age, and samples of ileum, thymus, bursa of Fabricius, spleen, liver, and muscle were collected for estimation of tissue O_2 consumption. Sections from each organ were dissected into 2 40-mg pieces and placed in separate respiration chambers containing 4 mL of M199 media at 37°C and an O_2 electrode (YSI, Yellow Springs, OH) with constant stirring, as previously described (Fan et al., 1997). Tissue O_2 consumption was expressed as μmol O_2/(min·g).

Isolation of Peripheral Blood Mononuclear Cells (PBMC)

Whole peripheral blood (~3 mL) was collected from 4 non-immunized broilers per diet group in heparinized syringes, equal volume of blood was pooled within treatment group, and the cells were separated (FicoLite LymphoH, specific density 1.077 g/mL; Atlanta Biologicals, Norcross GA). Density separated cells were then washed twice with ice-cold PBS and enumerated using a hemocytometer, and the cell concentration was adjusted to 10^6 cells/mL as previously described (Qureshi et al., 2000).

Analysis of PBMC ATP

Cellular ATP amounts were measured in white 96-well plates (CellTiter Glo luminescence ATP assay kit; Promega, Madison, WI). One hundred microliters of PBMC (10^5 cells per well) were seeded in 6 replicate wells and lysed, and ATP concentration was detected as relative light units (**RLU**) using a luminometer (Fluoroskan FL, Thermo Scientific, Hudson, NH). To assay for differences in the rate of ATP depletion, PBMC were seated in white 96-well plates at 10^5 cells/well in 6 replicate wells and incubated with the proton ionophore 2, 4-dinitrophenol (**DNP**, 5 mM; Sigma-Aldrich, St. Louis, MO), the ATP synthase inhibitor oligomycin (5 μg/mL, Sigma-Aldrich), or vehicle only (ethanol) at 42ºC for 0 and 15 min. The DNP or oligomycin specific change in ATP was calculated as the change in RLU over time with inhibitor (DNP or oligmycin$_{t0}$ – DNP or oligomycin$_{t15}$) minus the spontaneous change in RLU (Vehicle$_{t0}$ - Vehicle$_{t15}$).

Anti-Sheep Red Blood Cell ELISA

Soluble SRBC antigen was prepared as previous described (Kelly et al., 1979). Each well of a 96-well plate (NUNC MaxiSorp High Protein-Binding Capacity ELISA plates) was coated with 2 μg of SRBC protein in carbonate buffer (pH 9.6) and incubated overnight at 4°C. Serum samples from immunized and control broilers from both dietary treatments were diluted 1:50 in sample diluent (50 m*M* Tris buffered saline, pH 8.0, 1% BSA; 0.05% Tween 20) and incubated for 1 h at room temperature. Anti-SRBC IgM and IgG were detected using goat-anti-chicken IgM or IgG antibody (**Ab**), respectively, conjugated to horse radish peroxidase, and incubated for 1 h at room temperature, followed by 15 min incubation with substrate (Bethyl Laboratory, Inc., Montgometry, TX).

Mucosal IgA ELISA

The whole jejunal section of the small intestine (from the end of the duodenum to Meckel's diverticulum) was collected at 10, 14, 16, 21, and 28 d of age and flushed 4 times with 1 mL of PBS (pH 7.4) containing 0.1 mg/mL soybean trypsin inhibitor, 0.064 m*M* EDTA and 1 m*M* phenylmethanesulphonylfluoride (**PMSF;** Thermo Scientific, Rockford, IL). After collection, solid material was removed by centrifugation (at 650 × *g* for 10 min at 4°C), the supernatant was mixed with sodium azide (final concentration 1% wt/vol) and PMSF (final concentration, 1 m*M*), and further clarified by centrifugation at 27,000 × *g* for 20 min at 4°C as previously described (Elson et al., 1984). Total protein concentration was determined for each sample (BCA, Thermo Scientific, Rockford, IL), and analyzed for IgA using a chicken total IgA specific ELISA (Bethyl Laboratory, Inc.) and expressed on an equivalent protein basis.

Microarray Detection of Intestinal Gene Expression

At d 14, 6 non-immunized broilers with similar BW were selected from each treatment group and euthanized, and a 2-cm section of ileum (mid-point) was excised, rinsed with cold PBS, (pH 7.4), dissected longitudinally along the mesenteric line, immediately immersed in RNA Later (Ambion, Foster City, CA), and subsequently stored at −80°C until processed for RNA extraction. Tissues were then thawed and 50 mg sections excised and placed in 1 mL of TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH) with 0.3 mL of 0.5-mm glass beads. Tissues were disrupted at 4,800 oscillations/min for 30 s (Mini-Beadbeater; Biospec, Inc., Bartlesville, OK), a total of 3 times, with samples placed on ice for 1 min between cycles to avoid sample overheating. After disruption, samples were centrifuged at 1,000 × *g* for 4 min at 4°C and the supernatants were transferred to a new tube. The RNA was isolated by phenol/chloroform/isoamyl alcohol extraction as previously described (Druyan et al., 2008). The resulting RNA pellet was washed with 1 mL of 75% ethanol, air-dried, and then re-hydrated with 30 μL diethylpyrocarbonate-treated RNase free H_2O. Samples were, then, treated with DNase per the manufacturer's instructions (Ambion, Foster City, CA), DNA-free RNA samples were quantified using a ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE), and RNA integrity was verified by electrophoresis on 1.5% agarose gel.

Ten micrograms of RNA from each sample was prepared in 2 tubes (5 μg/tube) and incubated with random hexamers and oligo(dT) primers at 70°C for 10 min, chilled on ice for 2 min, complementary DNA (**cDNA**) synthesized using reverse transcriptase and aminoallyl-dNTP mix, and incubated for 60 min at 42°C following the manufacturer's instructions (Promega, Madison, WI). After cDNA synthesis, transcripts were fluorescently labeled by adding either Cy3-NHS or Cy5-NHS ester (GE Healthcare, Piscataway, NJ) to 1 of each tube/RNA sample. The cDNA transcripts were then column purified to remove unincorporated nucleotides and dye. The CyDye-labeled cDNA was quantified using a ND-1000 spectrophotometer (NanoDrop Technologies, Inc.), and the frequency of incorporation (**FOI**) was determined for each sample with each dye according to the equation: FOI = pmol of dye incorporated × 324.5/ng of cDNA. Only those samples with the FOI of 12 to 35 for Cy3 and 12 to 40 for Cy5 were used for hybridization.

Thirty picomoles of the Cy3- and Cy5-labeled cDNA probes were dried and resuspended in hybridization solution (Corning Inc., Corning, NY), applied to an array slide covered with a precleaned glass coverslip (Lifterslip, Portsmouth, NH), and hybridized for 16 h at 42°C. Microarray slides were scanned (ScanArray GX PLUS Microarray Scanner; PerkinElmer Life and Analytical Sciences, Shelton, CT). For each tissue and each treatment, there were 8 individually labeled cDNA.

Microarray Production

Microarrays were manufactured at North Carolina State University as described before (Druyan et al., 2008). Seventy oligonucleotides were designed for 514 unique gene sequences selected from the chicken and turkey genome using OligoWiz (www.cbs.dtu.dk/services/OligoWiz/) and manufactured (Operon Biotechnologies Inc., Germantown, MD). Oligonucleotides were printed (spotted) on slides (UltraGAPS Amino-Silane Coated Slides; Corning Inc., Acton, MA) using an arrayer (VersArray Chipwriter Compact Arrayer; Bio-Rad Inc., Waterloo, ON, Canada). Each gene was spotted a total of 12 times per array (technical replicates), dried for 24 h, and then cross-linked to the slides using a cross-linker (CL-1000 UV cross-linker; UVP Inc., Upland, CA) set to 6,000 × 100 μJ/cm^2.

Microarray data files were generated by extracting the intensity raw data for each slide and dye combination (ScanAlyze Software; Stanford University, Stanford, CA). Intensity data files were joined, transformed to a $\log_2$, and analyzed (JMP Genomics; SAS Inst. Inc., Cary, NC). Data normalization was performed using locally weighted regression and smoothing, first within array and then across all arrays. Evaluation of the normalization was monitored by distribution analysis of the transformed data. The resulting normalized $\log_2$ intensities were analyzed by using a mixed-model ANOVA (Wolfinger et al., 2001) with channel, treatment, and channel × treatment as fixed effects, and array as a

random effect. The resulting analysis of differentially expressed genes was not edited for global fold change, such as >2-fold, but, instead, left intact and the *P*-value threshold adjusted using the false discovery rate procedure ($P = 0.10$; Benjamini and Hochberg, 1995). This method of data filtering is especially useful in our experimental situation where subtle changes in gene expression are expected and was used in the development of the focused microarray as a tool for measuring small differences in gene expression below the detection limits of alternative procedures like real-time PCR (Druyan et al., 2007). Other studies have employed similar false discovery rate thresholds when looking at metabolic and nutritional experiments to detect small changes in gene expression (Tuggle et al., 2007). Biological processes were identified by using the approach of statistical overrepresentation in the database (Metacore database; GeneGo Inc., St. Joseph, MI), a Web-based application for identification of gene ontology processes associated with genes with difference in expression by the treatment and ranked according to their *P*-value as previously described (Nikolsky et al., 2005).

Real-Time Reverse Transcription PCR (RT-PCR)

The relative expression of IL-10, Myc, IL-12p40, GATA3, Bcl-6, and IL-12RB2 (FAM labeled TaqMan assays Gg03358688, Gg03355160, Gg03349680, Gg03370646, Gg03343577, and forward primer ATCAATGGGTATGTTGTGGAATGGA reverse primer ATGCGACAAGTCTTATCCAGCTT and probe CAGCCCGGAGCCCCAT, respectively; Life Technologies) in ileum of CSD and DFMD fed broilers was determined using the comparative Ct method. Briefly, DNA free total RNA was isolated from intestinal tissues from 3 unimmunized individual broilers/treatment group (RNeasy Mini, Qiagen). The RNA was then used as template to produce a cDNA library using random primers (High Capacity cDNA Reverse Transcription Kits; Life Technologies). Target gene expression was compared with the abundance of 18S ribosomal RNA (VIC-labeled TaqMan Ribosomal RNA Control Reagents; Life Technologies). Detection of both targets was performed in the same reaction (StepOnePlus Real-Time PCR System; Life Technologies), and the relative expression was calculated (StepOne Software v2.1; Life Technologies). One control sample was arbitrarily selected as the reference sample and the relative expression of the remaining samples calculated using the formula $2^{-\Delta\Delta Ct}$, and the average relative expression determined for each treatment group.

Statistical Analysis

The effects of direct fed microbials on whole body and organ O_2 consumption, relative organ weight, PBMC ATP amount, Ab production, and relative gene expression were determined using Student's *t*-test (Statistix 9, Analytical Software, Tallahassee, FL). Results are expressed as means ± SD. The pen was used as the experimental unit. The $P \leq 0.05$ was considered significant.

RESULTS

Energy Consumption

Over a 28-d period, there was no difference in BW or feed efficiency between broilers fed CSD or DFMD (data not shown). Additionally, there was no difference in whole body O_2 consumption, CO_2 expiration, or respiratory quotient of DFMD or CSD fed broilers (Figure 1).

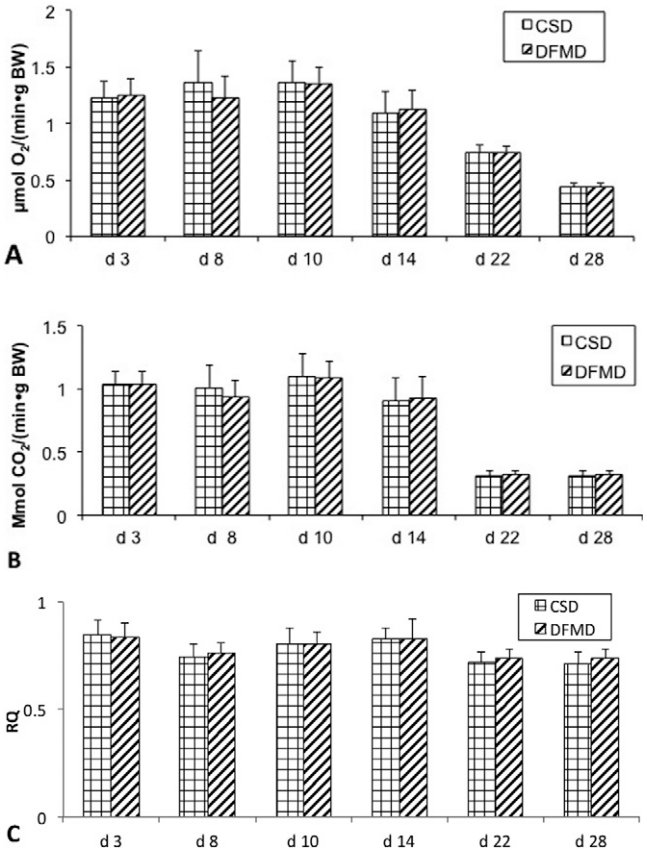

Figure 1. Effect of direct fed microbial supplementation (DFMD) and standard control starter diet (CSD) on whole body O_2 consumption (A), CO_2 expiration (B), and respiratory quotient (RQ; CO_2 expiration/O_2 consumption; C). Six broilers/time point from each treatment were selected for analysis, and data were adjusted on the basis of metabolic BW. Shaded bars represent the mean and error bars represent the SD. Data presented are representative of 5 independent experiments. There were no differences between the 2 treatments in any of the response criteria.

Table 2. Tissue O_2 consumption rate[1]

Item		CSD	DFMD	*P*-value
Thymus	d 8	1.172 ± 0.047	1.579 ± 0.094	0.034
	d 15	1.651 ± 0.086	1.923 ± 0.224	0.028
	d 22	1.781 ± 0.057	1.781 ± 0.061	0.289
Ileum	d 8	2.432 ± 0.218	1.601 ± 0.128	0.076
	d 15	2.6071 ± 0.302	1.823 ± 0.393	0.028
	d 22	2.514 ± 0.255	2.128 ± 0.206	0.021

[1]CSD = standard control starter diet, and DFMD = direct fed microbial diet; data are presented as mean μmol O_2/(min·g); n = 6.

Direct fed microbial-mediated changes in energy consumption of the ileum, thymus bursa of Fabricius, spleen, liver, and muscle were estimated at 8, 15, and 22 d of age, and the results indicated that the O_2 consumption rate of the ileum from DFMD broilers was decreased by 30 and 15% at 15 ($P = 0.028$) and 22 ($P = 0.021$) d of age, respectively (Table 2). Ileal O_2 consumption at d 8 was decreased numerically by 34% in the DFMD fed broilers as compared with controls ($P = 0.076$). The O_2 consumption rate of the thymus from DFMD fed broilers was increased by 35 and 16% at 8 ($P = 0.034$) and 15 ($P = 0.028$) d of age, respectively (Table 2). No other organs analyzed demonstrated a difference in O_2 consumption with direct fed microbial supplementation. It should be noted, however, that although there was no difference in O_2 consumption per unit mass of the bursa of Fabricus, the adjusted weight (mg/g BW) of the bursa from DFMD fed broilers was heavier than CSD fed broilers at 8 ($P = 0.008$) and 15 ($P = 0.023$) d of age (data not shown).

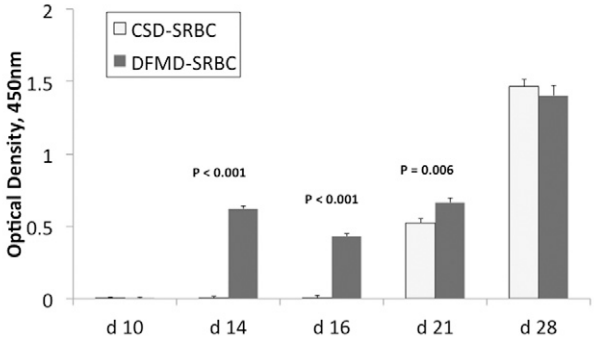

Figure 3. Effect of feeding direct fed microbial supplemented diet (DFMD) and standard control starter diet (CSD) on antigen specific serum IgG response. Broilers were vaccinated with sheep red blood cells (SRBC) at d 7 and boosted at 14 and 21 d of age. Serum samples were collected from 6 broilers/group and analyzed for the presence of anti-SRBC IgG by ELISA. Bars represent the mean optical density of replicate samples. Error bars represent the SD of the mean. Data presented is representative of 4 independent experiments. Broilers fed DFMD had increased antigen specific serum IgG response at d 14, 16, and 21.

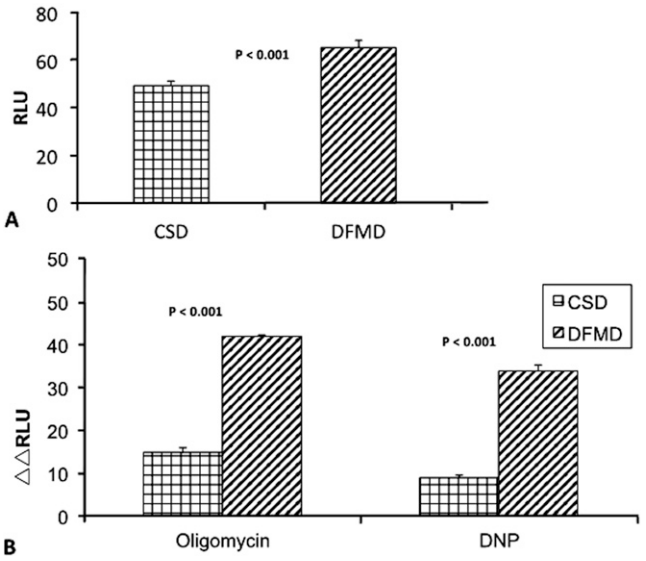

Figure 2. Effect of feeding direct fed microbial diet (DFMD) and standard control starter diet (CSD) on ATP concentration detected as relative light units (RLU) in peripheral blood mononuclear cell (PBMC) isolated from broilers fed experimental diets. Peripheral blood was collected from 4 CSD and 4 DFMD fed broilers at d 10. Equal volume of 4 replicate samples were pooled, PBMC isolated, and 10^5 cells/well in 6 replicate wells were assayed for the presence of ATP (A). To assay for differences in the rate of ATP turnover in each cell population, cells (10^5/well in 6 replicate wells) were treated with the ATPase inhibitor oligomycin (5 μg/mL), DNP (5 μ*M*), or vehicle alone for 15 min. The specific effect of ATPase inhibition was calculated by (RLU $drug_{t0}$ – RLU $drug_{t15}$) – (RLU $vehicle_{t0}$ – RLU $vehicle_{t15}$) for each PBMC source and inhibitor, and expressed as ΔΔ RLU (B). Bars represent the mean RLU of 6 replicate wells. Error bars represent the SD of the mean. Data presented are representative of 4 independent experiments. The PBMC isolated from broilers fed DFMD used increased amounts of ATP/cell than the PBMC isolated from those fed CSD.

ATP Content and Turnover in PBMC

The PBMC were isolated from unimmunized CSD and DFMD fed broilers and assayed for differences in the amount of ATP, and cells from DFMD fed broilers were found to have an increased concentration of ATP/cell as compared with CSD fed broilers ($P < 0.001$; Figure 2A). Isolated PBMC from DFMD fed and CSD fed broilers were then treated with ATP synthase inhibitors DNP or oligomycin for 15 min and assayed for differences in the loss of ATP, and these assays demonstrated a greater change in the amount of ATP/cell in DFMD fed broilers detected through a decrease in luciferase activity, as compared with CSD fed broilers ($P < 0.001$; Figure 2B).

Antigen Specific Immune Response

Serum was collected from SRBC immunized CSD and DFMD fed broilers and assayed for differences in the amount of SRBC-specific IgG, and DFMD fed broilers were found to produce anti-SRBC IgG within 7 d of immunization (d 14), whereas the CSD fed broilers had no detectable anti-SRBC IgG until 14 d post-immunization (d 21; $P < 0.001$; Figure 3). By 3 wk post-immunization

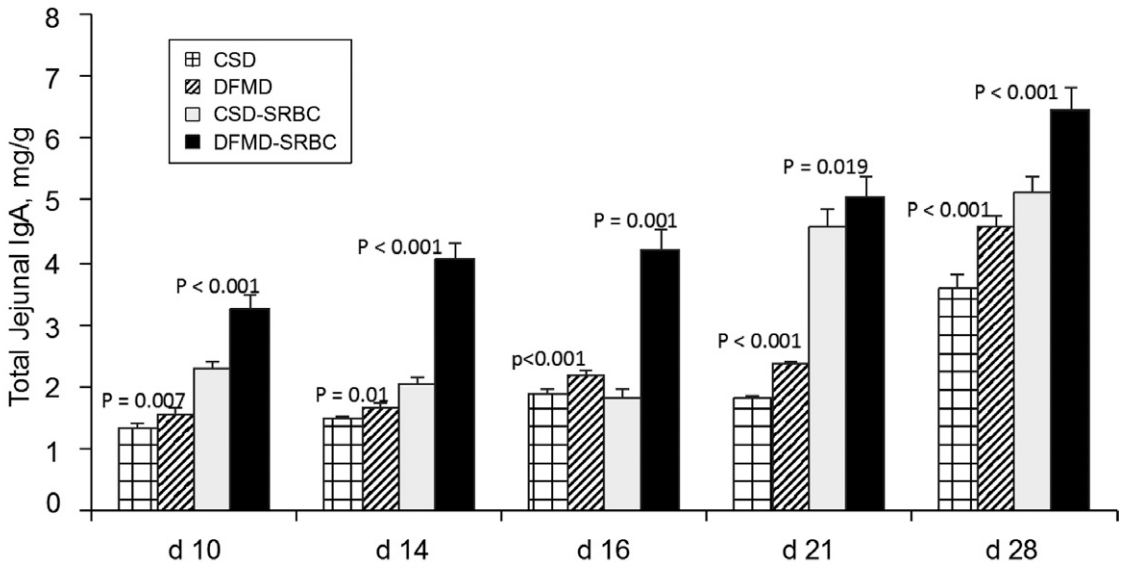

Figure 4. Effect of feeding direct fed microbial diet (DFMD) and standard control starter diet (CSD) on total IgA production. Sections of jejunum were collected at 10, 14, 16, 21, and 28 d of age, washed, and analyzed for amounts of IgA. Total IgA was detected by ELISA, quantitated using a standard curve, and normalized to total protein content of the sample. Data are presented as mean mg of IgA/g to total protein of replicate samples. Error bars represent the SD of the mean. The CSD and DFMD represent samples from unimmunized animals. The CSD-sheep red blood cells (SRBC) and DFMD-SRBC represent samples from immunized broilers. Data presented are representative of 4 independent experiments. Broilers fed direct fed DFMD had increased total IgA production.

(d 28) and for the remainder of the experiment, there was no difference in the concentration of anti-SRBC IgG between the 2 treatment groups.

Mucosal IgA Concentration

Jejunal sections were collected from SRBC immunized and unimmunized CSD and DFMD fed broilers, flushed with PBS, and the intestinal wash assayed for differences in the amount of total IgA, and intestinal wash samples from SRBC immunized DFMD fed broilers had greater concentrations of total IgA than the SRBC immunized CSD fed broilers at all time points post immunization ($P < 0.019$; Figure 4). Additionally, DFMD fed broilers were found to have more IgA in their intestine than CSD fed broilers without immunization ($P < 0.01$). Intestinal luminal washes from SRBC immunized broilers were assayed for antigen specific IgA; however, none was detected in either treatment group (data not shown).

Changes in Gene Expression

Sections of ileum were collected and analyzed for changes in gene expression associated with immune function using a targeted oligo-nucleotide array, and DFMD fed microbial supplementation was associated with decreased expression of 23 genes and increased expression of 7 genes as compared with CSD fed broilers (Table 3). The differentially expressed genes were then analyzed to identify known associations with signaling networks using the shortest path algorithms (Metcore), and this analysis identified the IL-27 signaling pathway as having the greatest relevance to the changes in gene expression detected in the targeted array. Changes in IL-27 associated gene expression were associated with increased expression of IL-10 and Myc and decreased expression of IL-12RB2, JAK2, NFκB, and CD28 (Figure 5).

Total RNA isolated from ileal sections was used to assess gene expression by real-time RT-PCR, and the results indicated that DFMD fed broilers had the reduced expression of IL-12p40 compared with those fed CSD ($P = 0.003$; Table 4). Additionally, real-time RT-PCR analysis demonstrated a numerical increase in expression of Myc ($P = 0.066$) and GATA3 ($P = 0.135$) in broilers fed DFMD compared with those fed CSD.

DISCUSSION

Previous studies in our laboratory have demonstrated that feeding broilers diets supplemented with direct fed microbials induces changes in the abundance and distribution of endogenous microbes (Chichlowski et al., 2007a) and affects the expression of inflammatory genes in the small intestine (Chichlowski et al., 2007b), and is associated with changes in the development of the small intestines along with changes in nutrient transport and fermentation products (Chichlowski et al., 2007a; Croom et al., 2009). Each of these observations speaks to one or more of the commonly proposed mechanisms for direct fed microbial enhanced performance, but no one observation alone explains how direct fed microbials promote health. Collectively these data, along with the data presented in the current study, indicate that the

Table 3. Ileal genes identified by focused array as differentially expressed in broilers fed the direct fed microbial diet as compared with those fed the standard control starter diet

Gene symbol	Fold change	*P*-value	Protein function
EGF	0.850	0.001	Epidermal growth factor is a potent mitogenic factor important in the growth, proliferation, and differentiation of numerous cell types (Herbst, 2004).
YWHAB	0.855	0.009	Belongs to the 14-3-3 family of proteins, members of which mediate signal transduction by binding to phosphoserine-containing proteins. May play role in linking mitogenic signaling to cell cycle machinery (Conklin et al., 1995).
STAT4	0.867	0.035	Member of the STAT family of transcription factors. After receptor binding by cytokines or growth factors or both, STAT family members are phosphorylated, form homo- or heterodimers, translocate to the nucleus, and act as transcription activators. STAT4 is essential in IL-12 signaling, and regulating the differentiation of T helper cells (Thierfelder et al., 1996)
IFNAR2	0.879	0.002	One of the 2 chains that make up the functional receptor for type-I interferon (Novick et al., 1994).
TNNT2	0.891	0.022	Tropomyosin-binding subunit of the troponin complex. Regulates muscle contraction in response to alterations in intracellular Ca+ concentration (Revera et al., 2007).
NFKB2	0.892	0.049	Nuclear factor NFκ-B p100 subunit is activated by cytokines, oxidant-free radicals, inhaled particles, ultraviolet irradiation, and pathogens. Activation is associated with inflammation and inflammatory disease (Chen et al., 1999).
CD28	0.916	0.031	Costimmulatory receptor expressed on T-cells. Required for T-cell activation (Linsley and Ledbetter, 1993).
COX10	0.918	0.038	Cytochrome c oxidase (COX), catalyzes electron transfer from reduced cytochrome c to oxygen during the mitochondrial respiratory chain (Murakami et al., 1997).
IGFBP2	0.926	0.035	Insulin-like growth factor binding protein 2. Found in cerebrospinal fluid, serum, milk, urine, synovial fluid, interstitial fluid, lymph follicular fluid, seminal fluid, and amniotic fluid. Inhibits IGF activity (Rajaram et al., 1997).
OGDH	0.929	0.010	One subunit of the 2-oxoglutarate dehydrogenase complex. Complex catalyzes conversion of 2-oxoglutarate (alpha-ketoglutarate) to succinyl-CoA and CO_2 during the Krebs cycle (Koike et al., 1992).
NOTCH1	0.935	0.009	Notch family member. Involved in developmental processes by regulating physically adjacent cells through controlling cell fate (Artavanis-Tsakonas et al., 1999).
MSX2	0.937	0.029	Member of the muscle segment homeobox gene family. Transcriptional repressor involved in controlling the fate of neural crest-derived cells required for proper craniofacial morphogenesis. May also be involved in promoting cell growth under other conditions (Semenza et al., 1995).
GPI	0.938	0.026	Glucose-6-phosphate isomerase. Enzyme that catalyzes the conversion of glucose-6-phosphate into fructose 6-phosphate in the second step of glycolysis. In the cytoplasm, GPI is involved in glycolysis and gluconeogenesis. Extracellular GPI is a neurotrophic factor for spinal and sensory neurons (Watanabe et al., 1996).
GAL6	0.940	0.019	β-defensins gallinacin-6. Defensins constitute a large family of small, cysteine-rich, cationic peptides implicated in the resistance of epithelial surfaces to microbial colonization (Xiao et al., 2004).
B4GALT1	0.942	0.019	One of seven β-1,4-galactosyltransferase (β4GalT) genes. Specific for the donor substrate UDP-galactose; all B4GalT members transfer galactose in a 1,4 linkage to similar acceptor sugars. β4GalT1 is unique among β4GalT enzymes as it participates in both glycoconjugate and lactose biosynthesis (Amado et al., 1999).
FHF-2	0.942	0.023	Fibroblast growth factor homologous factor-2. FHFs are a distinct group of the FGF family of ðbroblast growth factor signaling molecules involved in various developmental processes (Reed, 2002).
IL12RB2	0.943	0.012	Subunit of the IL-12 receptor complex. Expression of this gene is up-regulated by interferon-γ in Th1 cells, and plays a role in Th1 cell differentiation (Airoldi et al., 2002).
CASQ2	0.954	0.018	Calsequestrin is localized to the sarcoplasmic reticulum in cardiac and slow skeletal muscle cells. The protein is a calcium binding protein that stores calcium for muscle function (Slupsky et al., 1987).
MYBPC3	0.960	0.025	MYBPC3 is the cardiac isoform of myosin-binding protein C. Myosin-binding protein C is a myosin-associated protein found in the cross-bridge-bearing zone (C region) of A bands in striated muscle (Vikstrom and Leinwand, 1996).
CYGB	0.963	0.046	Cytoglobin is a ubiquitously expressed hexacoordinate hemoglobin thought to be involved in the diffusion of oxygen through tissues, scavenging nitric oxide and/or other reactive oxygen species, as well as protecting tissue from oxidative stress and hypoxia (Trent and Hargrove, 2002).
ADH5	0.965	0.038	Member of the alcohol dehydrogenase family. ADH5 has limited to no ethanol oxidation activity, but does oxidize long-chain primary alcohols and S-hydroxymethyl-glutathione. Plays an important role in the elimination of formaldehyde (Hur and Edenberg, 1992).
AGTR1	0.980	0.043	Type 1 receptor for angiotensin II. Binding to angiotensin II results in vasoconstriction, aldosterone synthesis and secretion, increased vasopressin secretion, cardiac hypertrophy, augmentation of peripheral noradrenergic activity, vascular smooth muscle cells proliferation, decreased renal blood flow, renal renin inhibition, renal tubular sodium reuptake, modulation of central sympathetic nervous system activity, cardiac contractility, central osmocontrol, and extracellular matrix formation. AGTR1 may also be involved in reperfusion arrhythmias (Catt et al., 1984).
JAK2	0.989	0.050	Janus kinase 2 is a protein tyrosine kinase associated with the type II cytokine receptor family (e.g. interferon receptors), the GM-CSF receptor family (IL-3R, IL-5R and GM-CSF-R), the gp130 receptor family (e.g., IL-6R, IL-27), and the single chain receptors (e.g. Epo-R, Tpo-R, GH-R, PRL-R) (Schindler et al., 2007).
SERPINH1	1.026	0.034	Also known as heat shock protein 47, SERPINH1 is a member of the serpin superfamily of serine proteinase inhibitors, is localized to the endoplasmic reticulum and plays a role in collagen biosynthesis as a collagen-specific molecular chaperone (Dafforn et al., 2001).
MYC	1.033	0.038	Multifunctional, transcription factor involved in cell cycle progression, apoptosis and transformation (Gearhart et al., 2007).
IL10	1.067	0.005	Cytokine produced primarily by monocytes. Broadly regarded as anti-inflammatory. Down-regulates the expression of Th1 cytokines, MHC class II Ags, and costimulatory molecules. Enhances B cell survival, proliferation, and antibody production (Pestka et al., 2004).
ATP1B1	1.082	0.048	Sodium/potassium-transporting ATPase subunit beta-1. Na+/K+-ATPase essential in regulating the electrochemical gradients of Na and K ions across the plasma membrane and thereby regulating osmolarity, sodium-coupled transport of organic and inorganic molecules, and electrical sensitivity of nerve and muscle cells (Lingrel et al., 1990).
ROR2	1.083	0.007	Receptor protein tyrosine kinase belonging to the ROR subfamily. Thought to be involved in formation of chondrocytes and possibly required for cartilage and growth plate development (Stricker et al., 2006).
PARD3	1.083	0.032	Partitioning defective 3 homolog is a member of the PARD family of proteins. PARD proteins are important in asymmetric cell division and polarized growth (Joberty et al., 2000).
PP2A	1.126	0.041	Protein phosphatase 2A is heterotrimeric protein phosphatase involved regulating cell signaling molecules such as Raf-1, MEK, and AKT (Ory et al., 2003).

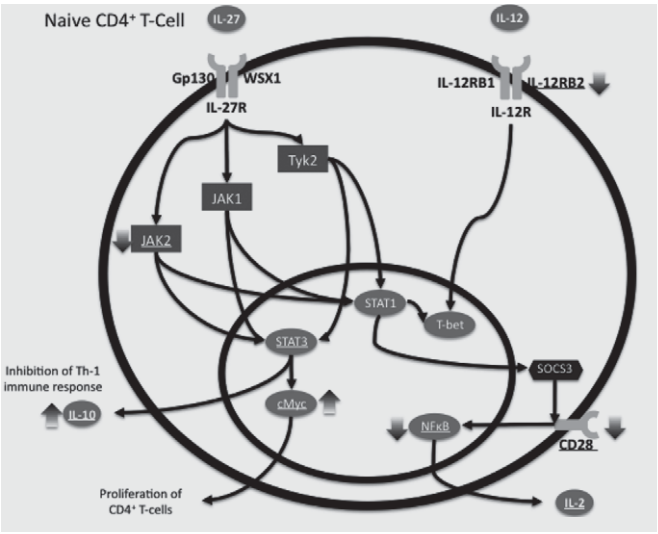

Figure 5. Diagram of IL-27 signaling pathway. Biological network analysis using the shortest path algorithm (GeneGo Inc., St. Joseph, MI) predicted the genes identified in the focused array analysis of ileum to have the greatest association with the IL-27 signaling pathway. Underlined genes denote those represented on the focused array. Those with gray block arrows denote genes with expression different between broilers fed microbial diet (DFMD) and standard control starter diet (CSD). Up arrows indicate expression in DFMD fed broilers > controls. Down arrows indicate expression in DFMD fed broilers < controls. Recognized interactions and associations between signaling pathway members are denoted by black arrow connectors. Data presented are representative of 2 independent experiments.

biological activities of direct fed microbials are achieved through a complex multi-variate system of mechanisms working in a coordinated manner.

The ability of the intestinal microflora to modulate host growth, digestion, and energetics has been demonstrated by several laboratories (Musso et al., 2011). Numerous studies comparing the growth performance of conventionally raised and germ free animals have demonstrated that colonized animals consume more energy and have increased fat deposits and glucose absorption (Backhed et al., 2004; Cani et al., 2009; Koliwad et al., 2009). In the present study, direct fed microbial supplementation did not result in changes in whole animal O_2 consumption; however, the difference in microflora between treatment groups was not as stark as conventional vs. germ free. Both treatment groups were colonized, and the amount of direct fed microbial specific organisms provided in the diet (approximately 10^5cfu/g) was approximately 1% of the total bacterial organisms found to exist naturally in the control feed. Furthermore, the total number of colony forming units supplemented represent approximately 1% of the total number of organisms found in the ileum and 0.0001% of the total organisms found in the cecum on a per gram basis (Salanitro et al., 1978). Although the amount of direct fed microbial organisms added to the feed represent a minor percentage of the total bacteria present in the intestine, differences were measured in the energy consumed by thymus and

Table 4. Direct fed microbial diet (DFMD) expression relative to standard control starter diet (CSD)

Gene	Fold change[1]	*P*-value
IL-10	1.09	0.426
IL-12p40	−3.31[2]	0.003
IL-12RB2	1.17	0.391
Myc	+1.61[3]	0.066
Bcl-6	1.00	0.483
GATA3	+1.55[3]	0.135

[1]Average fold change in gene expression of DFMD ileum relative to CSD.
[2]Expression was less in DFMD ileum relative to CSD.
[3]Expression was greater in DFMD ileum relative to CSD.

ileum, indicating direct fed microbial bacteria exert an influence on the bacterial ecosystem and host disproportionally to their percentage representation in the intestine (Maxwell et al., 1983).

Given the fact that energy consumption by the thymus increased and ileum decreased, it is tempting to conclude the energy saved in the ileum was reallocated to the immune system; however, the thymus was found to significantly increase its energy consumption before significant decreases were noted in the ileum. Whereas the difference in energy consumption at d 8 in the ileum did not meet the a priori significance level, the ileal samples from DFMD fed broilers were found to be 34% less than controls. When total size of the ileum relative to the thymus is considered, this energy savings could account for the increased consumption by the immune system; however, other metabolic processes and other organ systems may be involved.

At present, we hypothesize the introduction of direct fed microbial species interacts with the cells of the intestinal mucosa (epithelia, neuroendocrine, and mucosal intestinal cells). The result of this interaction is a change in the energy consumption of the small intestine, which then leads to more energy being available to the immune system, and the communication of signals to the systemic immune system to use those additional resources. Alternatively, the introduction of direct fed microbial organisms could first stimulate the systemic immune system that can lead to increased activity, energy consumption, and Ab production, resulting in reduced energy consumption by other tissues, most notably the small intestine. Understanding the specific order in which tissues and their energy consumption are affected by supplementation is critical to our ability to fully exploit direct fed microbials in food animal production. As such, elucidating the mechanism by which direct fed microbials affect the activity and energy consumed by the immune system is the primary focus of ongoing experiments in our laboratory.

As the "nursery" for T-lymphocytes, increased metabolic activity in the thymus indicates increased prolif-

eration or apoptosis or both as immature T-cells undergo selection to become mature, naïve T-cells and enter circulation. This is a very intriguing proposition, and experiments are currently underway to specifically address thymocyte activity in DFMD fed broilers. Because we were interested in understanding how direct fed microbial supplementation could increase broiler performance in the present study, we focused on determining if the increased energy expenditures in the thymus were associated with increased energy consumption by immune cells in circulation. Initial experiments to assess O_2 consumption by peripheral leukocytes were inconclusive. For this reason, the ATP assay was developed. The observation that PBMC from DFMD fed broilers consume more ATP/cell than CSD fed broilers indicates that whatever mechanism induced increased energy consumption by the thymus was acting on the immune system in general; however, additional experiments are needed to understand what cell population(s) in the PBMC is responsible for the increased ATP consumption.

Given the complex interactions among the microflora, mucosal barrier, and resident immune cells, relatively small changes in individual signaling molecules could result in a biologically significant constellation of signaling events. To best appreciate changes in signaling networks, the focused array followed by gene ontology analysis was used as the primary means to identify immune function associated changes in the intestine. In this approach, a targeted microarray, which included 12 replicate oligo spots/gene target, was selected. This represents a 6- to 12-fold increase in the number of technical replicates typically used in array assays, resulting in sensitivity equal to greater than that of real-time RT-PCR (Druyan et al., 2008). The results of the array analysis identified 30 genes with expression that differed between treatment groups. Many of these genes were associated with the immune system (STAT4, IFNAR2, NFκβ, CD28, IL-12RB2, Jack2, and IL-10). Of the immune associated genes, all but IL-10 were found to be down-regulated and all are associated with the Th1, pro-inflammatory, type of immune response (Trinchieri, 2003). Conversely, IL-10, which was up-regulated, is commonly regarded as an anti-inflammatory cytokine and associated with the Th2 type of immune response (Couper et al., 2008). Gene ontology analysis of the full complement of differentially regulated genes identified the changes in expression with that of the IL-27 signaling pathway and the balance between Th1 and Th2 immune responses. Interleukin-27 has presently not been identified in chickens, and, to our knowledge, this is the first report to suggest an IL-27-like signaling pathway may exist in birds.

To support the array and gene ontology results, 6 gene targets were selected for analysis by real-time RT-PCR. Three of the gene targets (IL-10, IL-12Rβ2, and Myc) were directly identified by the array as being differentially expressed. The remaining 3 gene targets (IL-12p40, GATA3, and Bcl-6) were not represented on the array but identified by the gene ontology analysis. Interestingly, the results of the real-time RT-PCR analysis do not support the array on a gene-by-gene basis; however, the overall impression of changes in signaling pathways does appear similar. Real-time RT-PCR did not demonstrate a difference in IL-10 or IL-12RB2 expression. The specific reason for this is presently unclear; however, given the fact that we have previously reported an increase in IL-10 expression in the ileum of direct fed microbial treated chicks (Chichlowski et al., 2007b), it is possible that the changes in expression are at or just below the limit of detection of the real-time RT-PCR assay. Real-time RT-PCR did indicate an increase in expression of Myc in DFMD treated broilers; however, this difference was not statistically significant at the a priori level of $P < 0.05$.

Interestingly, it is the 3 genes, analyzed on the basis of the gene ontology results, that provided the most support for the array data. The majority of the genes identified as down-regulated by the array are directly associated with the IL-12 cytokine and its signaling pathway. The real-time RT-PCR analysis of the IL-12p40 demonstrated a decrease in expression in DFMD treated broilers, again, indicating a possible shift from a Th1 to a Th2 type of immune response. If there was a shift towards more of a Th2 type of response, one would expect an increase in the expression of the transcription factor GATA3 and either no change or a decrease in expression of its repressor, Bcl-6 (Kusam et al., 2003). Analysis of these 2 genes by real-time RT-PCR indicated an increase in expression of GATA3 and no change in Bcl-6. Collectively, the changes in gene expression measured by these 2 gene transcription assays indicate a decrease in expression of Th1 type cytokines and a possible increase in a Th2 type of response, similar to other studies with direct fed microbials (Haghighi et al., 2008).

Interleukin-10 plays an important role in controlling inflammation in the intestine, which is best demonstrated through the use of IL-10 knockout mice as the experimental model of colitis (Madsen, 2001). Interleukin-10 is recognized to promote the differentiation of Th2 type $CD4^+$ T-cells and suppress the pro-inflammatory lineages Th1 and Th17 (Couper et al., 2008). The development of the Th2 lineage of $CD4^+$ T-cells results in the production of cytokines that promote antibody production by B-cells and antibody class switching (Cerutti, 2008). This putative decrease in ileal inflammatory status is supported by the targeted array, subsequent gene ontology, and real-time RT-PCR analysis that indicates a decrease in IL-12p40, IL-12RB2, CD28, and NFκB

expression, all of which are associated with Th1, Th17, and pro-inflammatory immune responses.

It is also interesting to note that several laboratories have reported a correlation between the numbers of Th17 T-cells and IL-17 expression and the presence of segmented filamentous bacteria (**SFB;** Ivanov et al., 2008). These poorly understood, unculturable bacteria have been found attached to epithelia cells of the ileum (Ivanov et al., 2009). The greater the number of SFB found in the intestine, the greater the number of Th17 cells and the greater the amount of inflammation and inflammation-related diseases (Ivanov et al., 2008; Miossec, 2009). Previous studies in our laboratory described a dramatic decrease in the number of SFB observed in the intestine of DFMD fed broilers as compared with controls (Chichlowski et al., 2007a).

The net result of increased IL-10, decreased IL-17, and promotion of Th2 type T-helper cells would be an enhanced humoral immune response, and after immunization with SRBC, the DFMD fed broilers produced detectable amounts of Ag specific IgG 9 d sooner than CSD fed broilers. If the challenge had been an infectious disease, the more rapid IgG response would likely mean the immune system of the DFMD fed broilers would clear the infection faster, minimizing the impact of disease on growth and performance (Aslam et al., 1998; Qureshi et al., 2000; Gross et al., 2002; Cheema et al., 2003; Wang et al., 2006; Adriaansen-Tennekes et al., 2009). It should be noted that within 14 d post-immunization (d 21), DFMD fed broilers had greater anti-SRBC IgG than CSD fed broilers; however, it is unclear if these differences are biologically significant, and ultimately by 21 d post-immunization, there was no difference in the amount of IgG between the groups. Additionally, there was no difference in the Ag specific IgM response (data not shown). This indicates the difference in the immune response between diets is a function of responsiveness, more rapid Ab class switching, and presumably T-cell help. This possible difference in T-cell help leading to Ab class switching was also demonstrated by the differences in total IgA in the jejunum. More total IgA was detected in the jejunal wash from DFMD fed broilers over CSD fed broilers. This difference was most pronounced after immunization, indicating the changes in the immune system, which resulted in increased IgA production, was amplified after immune stimulation. Although there are recognized T-cell independent mechanisms for increased mucosal IgA production, the similarities in the kinetics of increased IgA production after immunization compared with IgG production for both DFMD and CSD fed broilers indicates a potential role for helper T-cells.

Collectively, the results from the current study indicate that supplementation with direct fed microbials can affect the amount of energy consumed by different tissues, independent of any effect on growth, performance, or whole body energy consumption within the period of broiler growth studied. It is important to emphasize that the changes in energy and the tissues affected in the present study may be unique to the direct fed microbials employed and the biological activity in direct fed microbials and probiotics are very likely differ among genotypes and amounts given. The results of the present experiments are, to our knowledge, the first to indicate that direct fed microbials supplementation leads to increased energy expenditures by the immune system. This increased energy consumption is associated with increased immune activity, specifically more rapid production of Ag specific IgG and greater quantities of mucosal IgA. Ultimately, these data indicate the mechanism by which direct fed microbial supplementation promotes animal health is multifaceted and involves all the biological systems in the intestine to produce the overall phenotype. On the basis of these data, we hypothesize direct fed microbial modulation of the intestinal flora in our model results in reduced production of pro-inflammatory cytokines in the small intestine. This reduction in inflammation reduces the total amount of energy consumed in the intestine, and this energy is then consumed by the immune system, which, because of the reduced concentrations of pro-inflammatory cytokines, tends towards a Th2 type of immune response. As a result of these changes, broilers fed DFMD would then able to produce a more rapid and varied Ag specific Ab response and presumably result in the clearance of infections faster with reduced inflammation and, therefore, less impact on growth performance.

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