High-throughput gene expression analysis of intestinal intraepithelial lymphocytes after oral feeding of carvacrol, cinnamaldehyde, or Capsicum oleoresin

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ABSTRACT Among dietary phytonutrients, carvacrol, cinnamaldehyde, and Capsicum oleoresin are well known for their antiinflammatory and antibiotic effects in human and veterinary medicine. To further define the molecular and genetic mechanisms responsible for these properties, broiler chickens were fed a standard diet supplemented with either of the 3 phytochemicals and intestinal intraepithelial lymphocytes were examined for changes in gene expression by microarray analysis. When compared with chickens fed a nonsupplemented standard diet, carvacrol-fed chickens showed altered expression of 74 genes (26 upregulated, 48 downregulated) and cinnamaldehyde led to changes in the levels of mRNAs corresponding to 62 genes (31 upregulated, 31 downregulated). Most changes in gene expression were seen in the Capsicum-fed broilers with 98 upregulated and 156 downregulated genes compared with untreated controls. Results from the microarray analysis were confirmed by quantitative real-time PCR with a subset of selected genes. Among the genes that showed >2.0-fold altered mRNA levels, most were associated with metabolic pathways. In particular, with the genes altered by Capsicum oleoresin, the highest scored molecular network included genes associated with lipid metabolism, small molecule biochemistry, and cancer. In conclusion, this study provides a foundation to further investigate specific chicken genes that are expressed in response to a diet containing carvacrol, cinnamaldehyde, or Capsicum oleoresin.

Key words: chicken, microarray, gene expression, metabolism

INTRODUCTION

The use of antibiotics as growth promoters in feed has been criticized for the possible effects of agriculture-related antibiotics on the emergence of antibiotic resistance in human pathogens. Many countries are now producing significant amounts of poultry without antibiotic growth promoters even if the national regulation still authorizes their use. This evolution encouraged researchers to find alternative growth-promoting feed additives. Plant extracts are gaining interest as a feed additive (Greathead, 2003; Benchaar et al., 2008). The efficacy of such products has been demonstrated especially in poultry (Hernandez et al., 2004); however, their mode of action is still speculative. Many people argue a bactericide effect of essential oils known since ancient times, but the optimum dose of these products in vivo is much lower than those used in vitro. Therefore, the question remains of the mode of action of these extracts within the range of doses which are used for growth-promoting purposes. It is critical to obtain this knowledge to find the best way to utilize these additives.

The dietary effects of the mixture of 3 phytochemicals, carvacrol, cinnamaldehyde, and Capsicum oleoresin, as antibacterial and antifungal agents have been reported (Jamroz et al., 2005). Carvacrol is a component of numerous aromatic plants, such as Origanum vulgare, thyme, and wild bergamot (De Vincenzi et al., 2004). The antimicrobial functions of these herbs are associated with carvacrol (Burt et al., 2007). Cinnamaldehyde is a constituent of cinnamon and widely applied as flavoring. It has been proven to have strong antibacterial activity against Escherichia coli, Pseudomonas aeruginosa, Enterococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis, methicillin-resistant Staphylococcus aureus, Klebsiella pneumoniae, Salmonella sp., and Vibrio
**Materials and Methods**

### Experimental Birds and Diets

All experiments were approved by the Beltsville Agriculture Research Center Small Animal Care and Use Committee. One-day-old broilers (Ross/Ross, Longenecker’s Hatchery, Elizabethtown, PA) were housed in Petersime starter brooder units and randomly assigned to 4 groups (5 birds/group). Carvacrol, cinnamaldehyde, and *Capsicum* oleoresin were obtained from Pancosma S.A. (Geneva, Switzerland). *Capsicum* oleoresin (Pushp Brand Spices, Muninjhi Foods and Spices Pvt. Ltd., Indore, India) was extracted from crushed *Capsicum annuum* fruits with volatile solvents leading to an oleoresin and finally processed to produce a powder. Carvacrol and cinnamaldehyde were made by synthesis. All diets were formulated to meet the nutrient requirements for chickens as recommended by the NRC (1994). The proportions of each phytonutrient in feed were based on the optimal doses from our pretrial experiments in which these doses showed optimum protective immune response against oral coccidiosis challenge (unpublished data).

Chickens were fed for 7 d beginning from hatch with a standard diet alone (control) or with diets supplemented with carvacrol (5.0 mg/kg), cinnamaldehyde (3.0 mg/kg), or *Capsicum* oleoresin (2.0 mg/kg). The composition and the concentration of each phytonutrient in feed were shown in Table 1.

### RNA Extraction and Aminoallyl-Labeled RNA Preparation

After euthanization of the birds, intestines were cut longitudinally and washed 3 times with ice-cold Hanks’ balanced salt solution containing 100 U/mL of penicillin and 100 mg/mL of streptomycin (Sigma, St. Louis, MO). The mucosal layer of the entire intestine was carefully scraped using a surgical scalpel and IEL were isolated by Percoll density gradient centrifugation as described previously (Min et al., 2005). Total RNA was isolated from pooled IEL representing an equal number of cells (5.0 x 10^7 cells) per bird from each treatment group using Trizol (Invitrogen, Carlsbad, CA) and purified using the RNasy Mini RNA Purification Kit (Qiagen, Valencia, CA) according to the instruction of the manufacturer. In preliminary experiments, we determined that equal cell numbers and equal amounts of total RNA were obtained per unit volume of gut mucosa (D. K. Kim, unpublished data). Aminoallyl-labeled RNA from IEL was prepared using the Amino Alkyl Message Amp II aRNA Amplification Kit (Ambion, Austin, TX) according to the instruction of the manufacturer. Two 20-μg aliquots of each aminoallyl-RNA sample were fluorescently labeled with AlexaFlu-
or 555 or AlexaFluor 647 (Invitrogen) and labeled RNA were column-purified using the RNA Amplification Kit (Ambion). The RNA concentrations and labeling efficiencies were determined spectrophotometrically.

**Microarray Hybridization**

The avian IEL array (AVIELA) consisted of 10,162 spots representing elements from 3 sources: 1) cDNA from chicken-IEL (Min et al., 2005), 2) immune-related cDNA from the lipopolysaccharide-activated HD11 chicken macrophage cell line (Min et al., 2003), and 3) direct-PCR clones of selected chicken cytokines and chemokines (Min et al., 2005). Each element was duplicated on the array slide. Six microarray hybridizations were performed. According to a reference design with dye swap (Mcshane et al., 2003), 4 values were obtained for each treatment, 2 on one slide and 2 on the dye-swapped slide. Hybridizations were performed using HybIt hybridization buffer (TeleChem, Sunnyvale, CA) in ArrayIt reaction cassettes at 50°C overnight as described (Kim et al., 2008). After hybridization, the slides were rinsed in 0.5× saline sodium citrate (SSC), 0.01% SDS at room temperature and washed once for 15 min in 0.2× SSC, 0.2% SDS at 50°C, 3 times for 1 min in 0.2× SSC at room temperature, and 3 times for 1 min in distilled water at room temperature. Each sample had a repeated hybridization using the alternate fluorescent dye between the treatment and control.

**Microarray Scanning and Image Analysis**

Images were acquired by laser confocal scanning using a ScanArray Lite microarray analysis system (Perkin-Elmer, Boston, MA) at a resolution of 10 μm. A 16-bit TIFF image was generated for each channel corresponding to the Alexa Fluor 555 and Alexa Fluor 647 dyes. The scanned microarray images for each channel were overlaid and fluorescent intensities were quantified using ScanArray Express version 3.0 software (Perkin-Elmer). Spots were detected using an adaptive circle algorithm in the ScanArray program and all spots were visually confirmed.

**Microarray Data Analysis**

GeneSpring GX 7.3 software (Silicon Genetics, Redwood, CA) was used to qualify and normalize image analysis data and to perform the fold-change analyses. Median signal intensities were qualified by subtracting the median local background and normalized by block locally weighted regression and smoothing scatter plots methods. Flag information was applied to filter bad spots with genes missing more than 50% of their values because of a bad signal-to-noise ratio being removed. To generate signal ratios, signal channel values (treatment group) were divided by control channel values (control group). The significantly differentially ex-
pressed genes were filtered using the volcano plot built by comparing the treatment of each phytonutrient with itself. This volcano plot method is a statistically rigorous approach to quantifying microarray expression data that allows the relative effects of multiple treatments to be compared and incorporates analytical methods that are common to quantitative genetics (Jin et al., 2001). The modulated elements were defined by 2-fold differences and a cutoff of \( P < 0.05 \) by parametric test. The microarray information has been submitted online into the Minimum Information About a Microarray Experiment (http://www.mged.org/Workgroups/MIAME/). The accession number for this study is E-MEXP-2204.

### Bioinformatics Analysis

All sequence data files were obtained from the compiled database of the National Center for Biotechnology Information (NCBI). The IEL cDNA elements that were used to create the IEL cDNA microarray were mapped to the chicken genome reference assembly (version 2.1) and reference RNA and protein sequences (formatted database for BLAST, May 2006) using NCBI BLAST (version 2.2.13). To analyze pathway information, chicken Entrez gene identification numbers (ID) that were differentially expressed >2.0-fold between the treatment and control groups were mapped to Homolo-

### Table 3. Gene expression profiles induced by phytonutrients

<table>
<thead>
<tr>
<th>Dietary supplement</th>
<th>Upregulated</th>
<th>Downregulated</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carvacrol (5 mg/kg)</td>
<td>26</td>
<td>48</td>
<td>74</td>
</tr>
<tr>
<td>Cinnamaldehyde (3 mg/kg)</td>
<td>31</td>
<td>31</td>
<td>62</td>
</tr>
<tr>
<td>Capsicum annuum oleoresin (2 mg/kg)</td>
<td>98</td>
<td>156</td>
<td>254</td>
</tr>
</tbody>
</table>

1Chicken intraepithelial lymphocyte (IEL) transcripts altered >2.0-fold using the avian IEL array after dietary supplementation and compared with a nonsupplemented diet.
gene ID (locus link ID) for human genes because a large portion of these sequences have not been defined in chicken. The mapped human gene ID were used for the classification by the terms of Gene Ontology (GO) from the PANTHER database (http://www.pantherdb.org). Annotation derived from orthologous human genes allows for cross-species comparisons between chicken and human array data (Smith et al., 2006).

The networks of genes that were differentially expressed by dietary Capsicum oleoresin were analyzed by the Ingenuity Pathways Analysis software (Ingenuity Systems, Redwood City, CA). The data set containing gene identifiers mapped to Homologene ID and corresponding expression values were uploaded into the application. Each identifier was mapped to its corresponding gene object in the Ingenuity knowledge base. A fold-change cutoff of 2.0 was set to identify genes whose expression was significantly differentially regulated. These genes, called focus genes, were overlaid onto a global molecular network developed from information contained in the Ingenuity knowledge base.

Networks of focus genes were algorithmically generated based on their connectivity.

**Quantitative Real-Time PCR**

To confirm gene expression changes observed by microarray analysis, quantitative real-time PCR (qRT-PCR) was performed as described (Hong et al., 2006). Equivalent amounts of the same RNA samples used for microarray hybridizations were reverse-transcribed using the StrataScript First Strand Synthesis System (Stratagene, La Jolla, CA). Amplification and detection were carried out with the Mx3000P system and Brilliant SYBR Green qRT-PCR master mix (Stratagene). Standard curves were generated using log_{10}-diluted standard RNA and levels of individual transcripts were normalized to those of glyceraldehyde 3-phosphate dehydrogenase by the Q-gene program (Muller et al., 2002). For the calculation of fold changes between control and treatment groups, the normalized cycle threshold values of the target gene to glyceraldehyde 3-phos-

![Figure 2](image-url)
Figure 3. Gene Ontology (GO) analysis of genes mapped for human exhibiting >2.0-fold up- and downregulated expression after 7 d of dietary supplementation with Capsicum oleoresin. The GO terms were based on the PANTHER databases (http://www.pantherdb.org).

Table 4. Differentially expressed genes related to metabolism after dietary supplementation with carvacrol compared with nontreated controls

<table>
<thead>
<tr>
<th>Gene Ontology category</th>
<th>Gene name</th>
<th>Gene symbol</th>
<th>Score</th>
<th>Human gene ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrate metabolism</td>
<td>Protein phosphatase 1, regulatory (inhibitor) subunit 3B</td>
<td>PPP1R3B</td>
<td>8.00E-106</td>
<td>79660</td>
</tr>
<tr>
<td>Lipid, fatty acid, and steroid metabolism</td>
<td>Phospholipase A2, group IIE</td>
<td>PLA2G2E</td>
<td>0.00E+00</td>
<td>10154</td>
</tr>
<tr>
<td>Nucleoside, nucleotide, and nucleic acid metabolism</td>
<td>Transcription factor 2, hepatic; LP-B3; variant hepatic nuclear factor</td>
<td>TCF2</td>
<td>0.00E+00</td>
<td>6928</td>
</tr>
<tr>
<td>Phosphorylation</td>
<td>SET domain containing 1B</td>
<td>SETD1B</td>
<td>0.00E+00</td>
<td>22067</td>
</tr>
<tr>
<td>Solute carrier family 34 (sodium phosphate), member 2</td>
<td>SLC34A2</td>
<td>SL34A2</td>
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<tr>
<td>Selenoprotein X, 1</td>
<td>Selenoprotein X, 1</td>
<td>SEPX1</td>
<td>2.00E-32</td>
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<tr>
<td>Dual specificity phosphatase 1</td>
<td>Dual specificity phosphatase 1</td>
<td>DUSP1</td>
<td>0.00E+00</td>
<td>1843</td>
</tr>
<tr>
<td>Protease, serine, 2 (trypsin 2)</td>
<td>Protease, serine, 2 (trypsin 2)</td>
<td>PRSS2</td>
<td>0.00E+00</td>
<td>5646</td>
</tr>
<tr>
<td>Serine-threonine kinase 25 (STE20 homolog, yeast)</td>
<td>Serine-threonine kinase 25 (STE20 homolog, yeast)</td>
<td>STK25</td>
<td>0.00E+00</td>
<td>10494</td>
</tr>
<tr>
<td>Other metabolism</td>
<td>Coenzyme Q9 homolog, methyltransferase (Saccharomyces cerevisiae)</td>
<td>COQ9</td>
<td>0.00E+00</td>
<td>84274</td>
</tr>
<tr>
<td>Ornithine decarboxylase antizyme 1</td>
<td>Ornithine decarboxylase antizyme 1</td>
<td>OAZ1</td>
<td>0.00E+00</td>
<td>4946</td>
</tr>
<tr>
<td>Serine-threonine kinase 25 (STE20 homolog, yeast)</td>
<td>Serine-threonine kinase 25 (STE20 homolog, yeast)</td>
<td>STK25</td>
<td>0.00E+00</td>
<td>10494</td>
</tr>
</tbody>
</table>

1Gene names for human mapped by chicken Entrez gene identification numbers.
2ID = identification number.
phate dehydrogenase were calibrated to the relevant control line values. Oligonucleotide primers for qRT-PCR are listed in Table 2. Each analysis was performed in triplicate.

RESULTS

Gene Expression Profiles Induced by Phytonutrients

Compared with untreated controls and using a cut-off of >2.0-fold differential expression, the levels of 74 IEL mRNA were altered in carvacrol-fed chickens (26 upregulated, 48 downregulated), whereas cinnamaldehyde-fed birds exhibited changes in 62 transcripts (31 upregulated, 31 downregulated). The birds given the Capsicum oleoresin-supplemented diet displayed altered expression of 254 genes (98 upregulated, 156 downregulated; Table 3). The genes changed by each dietary supplementation of carvacrol, cinnamaldehyde, and Capsicum oleoresin are listed in Supplemental Tables 1, 2, and 3, respectively (http://ps.fass.org/content/vol89/issue1/).

GO Annotation and Bioinformatics Analysis

The altered transcripts corresponding to human gene ID were analyzed by the GO Index using PANTHER database (Mi et al., 2005; Figures 1, 2, and 3). Of the transcripts corresponding to genes classified in the biological process branch, 76 were related to metabolism (carvacrol, 13; cinnamaldehyde, 12; Capsicum oleoresin, 51; Tables 4, 5, and 6). In the specific category of protein metabolism, 24 genes were modulated (carvacrol, 4; cinnamaldehyde, 3; Capsicum oleoresin, 17). In the category of signal transduction, 15 genes were altered (5, 1, and 9, respectively); in the category of immunity and defense-related processes, 24 genes were modulated (2, 5, or 17, respectively); and in the category of nucleoside, nucleotide, and nucleic acid metabolism, 24 genes were modulated (2, 5, or 17, respectively).

Pathway Analysis of the Network Genes

Because the greatest number of alterations in IEL transcript levels was seen after the Capsicum oleoresin-supplemented diet, further pathway analysis was performed using the Ingenuity knowledge base to identify the biological functions that were most significant to the genes in the network. For this analysis, the genes were matched with human gene counterparts and the human gene ID were used as the data set. In this analysis, all relationships between genes in the network are graphically represented as lines and nodes that are displayed using various shapes representing the functional class of the gene product. These relationships are supported by at least one literature reference or from canonical information stored in the Ingenuity knowledge base. A total of 10 biologically relevant networks were determined in this study. The most significant network was composed of 35 focus genes [acyl-coenzyme A oxidase 1, palmitoyl (ACOX1); adipose differentiation-related protein (ADFP); adiponectin (ADIPQ); adiponectin receptor 2 (ADIPOR2); cyclophilin 1 (CND1); CD36 molecule (CD36); CD74 molecule (CD74); CDC5 cell division cycle 5-like (Schizosaccharomyces pombe) (CDC5L); collagen; cystatin C (amyloid angiopathy and cerebral hemorrhage) (CST3); cathepsin S (CTSS); eukaryotic translation elongation factor 2 (EEF2); extracellular signal-regulated kinase (ERK); fatty acid synthase (FASN); hexokinase (HK1); insulin; kinesin family member 5B (KIF5B); low-density lipoprotein (LDL); lectin, galactoside-binding, soluble, 3 (galactinin).

Table 5. Differentially expressed genes related to metabolism following dietary supplementation with cinnamaldehyde compared with nontreated controls

<table>
<thead>
<tr>
<th>Gene Ontology category</th>
<th>Gene symbol</th>
<th>Score</th>
<th>Human gene ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrate metabolism</td>
<td>UDP glycosyltransferase 8 (UDP-galactose ceramide galactosyltransferase)</td>
<td>UCT8</td>
<td>7368</td>
</tr>
<tr>
<td>Carbohydrate metabolism</td>
<td>Oxoglutarate (α-ketoglutarate) dehydrogenase (lipoamide)</td>
<td>OGDH</td>
<td>4567</td>
</tr>
<tr>
<td>Carbohydrate metabolism</td>
<td>UDP glycosyltransferase 8 (UDP-galactose galactosyltransferase)</td>
<td>UCT8</td>
<td>7368</td>
</tr>
<tr>
<td>Lipid, fatty acid, and steroid metabolism</td>
<td>DEAD (Asp-Glu-Ala-Asp) box polypeptide 1</td>
<td>DDX1</td>
<td>1653</td>
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<tr>
<td>Lipid, fatty acid, and steroid metabolism</td>
<td>Phosphotyrosine phosphatase 6C, cGMP-specific (cGMP-specific phosphatase 6C)</td>
<td>PTP6C</td>
<td>5146</td>
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<td>Lipid, fatty acid, and steroid metabolism</td>
<td>Chromobox homolog 3 (HP1 gamma homolog, Drosophila) (CHROMBOX3)</td>
<td>CBX3</td>
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<td>Lipid, fatty acid, and steroid metabolism</td>
<td>CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) phosphatase, subunit 1</td>
<td>CTDP1</td>
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<tr>
<td>Lipid, fatty acid, and steroid metabolism</td>
<td>SMYD family member 5</td>
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<td>Protein metabolism</td>
<td>Serine-threonine kinase 4</td>
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<td>6789</td>
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<tr>
<td>Protein metabolism and modification</td>
<td>Nucleotide binding protein 2 (Mr1D homolog, Escherichia coli)</td>
<td>NUP122</td>
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<td>Protein metabolism and modification</td>
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<tr>
<td>Protein metabolism and modification</td>
<td>Serine-threonine kinase 4</td>
<td>STK4</td>
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<tr>
<td>Protein metabolism and modification</td>
<td>Serine-threonine kinase 4</td>
<td>STK4</td>
<td>6789</td>
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</table>

1Gene names for human mapped by chicken Entrez gene identification numbers.
2ID = identification number.
Table 6. Differentially expressed genes related to metabolism after dietary supplementation with Capsicum oleoresin compared with nontreated controls

<table>
<thead>
<tr>
<th>Gene Ontology category</th>
<th>Gene name</th>
<th>Gene symbol</th>
<th>Score</th>
<th>Human gene ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acid metabolism</td>
<td>Sphingosine-1-phosphate lyase 1</td>
<td>SCP1L</td>
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<td>Fatty acid synthase</td>
<td>FASN</td>
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<td>2194</td>
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<td>Carbohydrate metabolism</td>
<td>Chitinase, di-N-acetyl-</td>
<td>CTBS</td>
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<td>Protein phosphatase 1, regulatory (inhibitor) subunit 3B</td>
<td>PP1IR3B</td>
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<td>Hexokinase 1</td>
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<td>Oxoglutarate (α-ketoglutarate) dehydrogenase (lipoamide) (OGDH)</td>
<td>OGDH</td>
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<td></td>
<td>Adiponecin, C1Q and collagen domain containing 1</td>
<td>ADIPOQ</td>
<td>0.00E+00</td>
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<td>N-myc downstream regulated gene 1 (NDRG1)</td>
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<td>Amino acid metabolism</td>
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<td>Fatty acid synthase</td>
<td>FASN</td>
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<td>Pyruvate dehydrogenase</td>
<td>PDP3</td>
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<td>Acetyl-CoA synthetase</td>
<td>ACS1</td>
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<tr>
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<td>Cytokine receptor 2</td>
<td>CTSS</td>
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<td>Cytosol 1, family 2, subfamily J, polypeptide 2</td>
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<td>CDK5 cell division cycle 5-like (Schizosaccharomyces pombe)</td>
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<td>Transcription elongation regulator</td>
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<td>Exosome component 1</td>
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<td>ZMYND11</td>
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<td>V-maf musculoaponeurotic fibrosarcoma oncogene homolog B (avian)</td>
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<td>PAX5 interacting protein (pre-RNA, subunit 2, 64 kDa, Tau variant)</td>
<td>CSTF2T</td>
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<td>DEAD (Asp-Glu-Ala-Asp) box polypeptide 15</td>
<td>DDX12</td>
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<td>8866</td>
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<td>Poly(A) polymerase</td>
<td>PAPOLA</td>
<td>0.00E+00</td>
<td>10914</td>
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<td>Serine-threonine kinase 25 (STE20 homolog, yeast)</td>
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<td></td>
<td>Unc-51-like kinase 1 (Caenorhabditis elegans)</td>
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<td>EIF2</td>
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<td>5045</td>
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<td>ATG4B</td>
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<td>23192</td>
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<td>Ubiquitin-conjugating enzyme E2I (UBC9 homolog, yeast)</td>
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<td>Protease, serine, 2 (trypsin 2)</td>
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<td>EIF3F6IP</td>
<td>0.00E+00</td>
<td>5198</td>
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<td>RPL0</td>
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<td>Procollegens-lysine 1, 2-oxoglutarate 5-dioxygenase 1</td>
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<td>531</td>
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<td>Carboxypeptidase S</td>
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<td>Threonyl-tRNA synthetase</td>
<td>TARS</td>
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<td>CHP</td>
<td>7.00E-120</td>
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</table>

1 Gene names for human mapped by chicken Entrez gene identification numbers.

2 ID = identification number.
regulated by 17 focus genes with a score of 31, was related with cellular assembly and organization (P-value: 3.46 × 10^{-4} to 2.94 × 10^{-8}); gastrointestinal disease (P-value: 3.50 × 10^{-5} to 2.36 × 10^{-2}); and genetic disorder (P-value: 2.56 × 10^{-3} to 2.94 × 10^{-7}) (Figure 5). The second network of genes included actin, V-akt murine thymoma viral oncogene homolog (Akt); activator protein-1 (Ap1); caspase; CD247 molecule (CD247); calcium binding protein P22 (CHP); cAMP response element binding (Creb); colony stimulating factor 3 receptor (granulocyte) (CSF3R); cytochrome P450, family 2, subfamily J, polypeptide 2 (CYP2J2); Fas (TNFRSF6)-associated via death domain (FADD); follicle-stimulating hormone (FSH); histone H3; histone H4; hematological and neurological expressed 1 (HN1); interferon-α; c-Jun N-terminal kinase (JNK); mitogen-activated protein kinase (MAPK); MYST histone acetyltransferase 2 (MYST2); nuclear factor of activated T-cells (NFAT); nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB); phosphoinositide 3-kinase (PI3K); prostaglandin-endoperoxide synthase 2 (PTGS2); Ras protein (Ras); RHOG = Ras homology growth-related; RNA polymerase II; splicing factor 3b, subunit 4 (SF3B4); splicing factor proline/glutamine-rich (polypyrimidine tract binding splicing factor 3b, subunit 4) (SF3B); spliceosome 72 kDa (5RP72); serine-threonine kinase 25 (STK25); spleen tyrosine kinase/zetakain-associated protein kinase; transcription elongation regulator 1 (TCERG1); ubiquitin-conjugating enzyme E2I (UBE2I); ubiquitin; WAS/WASL interacting protein family, member 1 (WIPF1); and zyxin (ZYX).

**qRT-PCR**

To confirm the microarray analysis, we selected 7 genes and followed the kinetics of their corresponding transcript levels after dietary supplementation with **Capsicum oleoresin**. All of the selected genes showed >2.0-fold altered expression in the normalized AVIELA data (P < 0.05). Of these, 2 (CD74 and CDC5L) were associated with the first network and 3 (UBE2I, CD247, and FADD) were included in the second network of pathway analysis. As shown in Figure 6, the transcriptional changes in these genes as assessed by qRT-PCR showed similar patterns when compared with the original microarray data.

**DISCUSSION**

The AVIELA microarray used in this study was originally described for its ability to characterize gene expression in IEL of chickens infected with the intestinal apicomplexan protozoa Eimeria, the etiologic agent of avian coccidiosis (Min et al., 2003, 2005; Kim et al., 2008). Because the mucosal layer of the intestine not only plays an important role in the immune defense against ingested pathogens but also comes in direct contact with foods and nutrients, we used the AVIELA as a surrogate tool for gene expression profiling during digestive and absorptive processes in the chicken gut. The utility of this approach was verified by the fact that the results from the microarray analysis were confirmed by qRT-PCR. The small differences in the magnitude of the changes observed by microarray and qRT-PCR might be due to differences of the normalization methods used by the 2 approaches or the different fluorescent dyes used, or both (Lee et al., 2002).

This trial demonstrated that 5 mg/kg of carvacrol altered expression of 74 genes (26 upregulated, 48 downregulated), 3 mg/kg of cinnamaldehyde led to changes in the expression of 62 genes (31 upregulated, 31 downregulated), and 2 mg/kg of Capsicum oleoresin led to alterations in 254 genes expression (98 upregulated, 156 downregulated) compared with untreated controls. These results suggest that among these 3 phytonutrients, Capsicum oleoresin is the strongest modulator of transcriptional control in chicken IEL.

**Table 7. Differentially expressed genes related to immunity and defense after dietary supplementation with carvacrol or Capsicum oleoresin compared with nontreated controls**

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Gene name 1</th>
<th>Gene symbol</th>
<th>Score</th>
<th>Human gene ID 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carvacrol</td>
<td>Selenoprotein X, 1</td>
<td>SBP1X</td>
<td>2.00E-32</td>
<td>51754</td>
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<tr>
<td></td>
<td>Chemo kinase (C motif) ligand 2</td>
<td>XCL2</td>
<td>6.00E-05</td>
<td>6546</td>
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<tr>
<td></td>
<td>Leucine-rich repeat containing 59</td>
<td>LRRC59</td>
<td>0.00E+00</td>
<td>55379</td>
</tr>
<tr>
<td></td>
<td>CD5 molecule</td>
<td>CD5</td>
<td>0.00E+00</td>
<td>921</td>
</tr>
<tr>
<td>Capsicum oleoresin</td>
<td>Lectin, galactoside-binding soluble, 3 (galectin 3)</td>
<td>LGALS3</td>
<td>7.00E-09</td>
<td>30528</td>
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<tr>
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<td>Chromosome 9 open reading frame 58</td>
<td>CHORF55</td>
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<td>TAP binding protein (topasin)</td>
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<td>Colony stimulating factor 3 receptor (granulocyte)</td>
<td>CSF3R</td>
<td>0.00E+00</td>
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<tr>
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<td>CD74 molecule, MHC, class II invariant chain</td>
<td>CD74</td>
<td>0.00E+00</td>
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<td>Cathepsin S</td>
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<td>Translin</td>
<td>TSN</td>
<td>0.00E+00</td>
<td>7247</td>
</tr>
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<td></td>
<td>Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)</td>
<td>PTGS2</td>
<td>0.00E+00</td>
<td>5743</td>
</tr>
</tbody>
</table>

1Gene names for human mapped by chicken Entrez gene identification numbers.
2ID = identification number.
**Capsicum** oleoresin is obtained from pepper plants as a mixture containing capsaicinoids, including the active ingredient capsaicin, and other compounds. Furthermore, capsaicin has been reported to influence various metabolic processes (Miller et al., 1983). Capsaicin and capsaicinoids have been reported for their effects on the gastrointestinal tract and animal nutrition (Nopanitaya, 1974). Sambaiah and Satayanarayana (1989) have postulated that capsaicinoids counteract the accumulation of fat in the liver by the reduction of hepatic lipogenesis or increased oxidation of lipids, or both. Capsaicin also induces neurogenic inflammation, has analgesic and anti-inflammatory activities, and inhibits the progression of early or late stage tumors (Beltran et al., 2007). Finally, *Capsicum* oleoresin increased pancreatic and intestinal enzyme activity (Platel and Srinivasan, 2000) and bile flow and bile acid secretion (Abdel Salam et al., 2005).

Within the 254 genes altered by feeding *Capsicum* oleoresin, the most significant biologically relevant network activated by *Capsicum* oleoresin intake was composed of 35 focus genes that encode for gene products related to lipid metabolism, small molecule biochemistry, and cancer. In chickens, capsaicin, cinnamaldehyde, and carvacrol induced lipase activity in the pancreas and...
Figure 5. Second network of genes exhibiting >2.0-fold up- and downregulated expression levels after 7 d of dietary supplementation with *Capsicum* oleoresin. The intensity of each gene indicates the expression level of the genes. Up- and downregulated genes are illustrated with red and green colors, respectively. ZYX = zyxin; RHOG = Ras homology growth-related; CYP2J2 = cytochrome P450, family 2, subfamily J, polypeptide 2; CHP = calcium binding protein P22; HN1 = hematological and neurological expressed 1; FADD = Fas (TNFRSF6)-associated via death domain; SRP72 = signal recognition particle 72 kDa; JNK = c-Jun N-terminal kinase; Akt = V-akt murine thymoma viral oncogene homolog; MAPK = mitogen-activated protein kinase; SFPQ = splicing factor proline/glutamine-rich (polypurine tract binding protein associated); NF-κB = nuclear factor κ-light-chain-enhancer of activated B cells; NFAT = nuclear factor of activated T-cells; CSF3R = colony stimulating factor 3 receptor (granulocyte); PI3K = phosphoinositide 3-kinase; PTGS2 = prostaglandin-endoperoxide synthase 2; Apol = activator protein 1; Creb = cAMP response element binding; TCERG1 = transcription elongation regulator 1; SF3B4 = splicing factor 3b, subunit 4; WIPF1 = WAS/WASL interacting protein family, member 1; FSH = follicle-stimulating hormone; CD247 = CD247 molecule; STK25 = serine-threonine kinase 25; MYST2 = MYST histone acetyltransferase 2; UBE2I = ubiquitin-conjugating enzyme E2I. Ras = Ras protein; SYK/ZAP = SYK/ZAP = spleen tyrosine kinase/zeta-chain-associated protein kinase.

...intestine wall (Jamroz et al., 2005). Therefore, it was suggested that they played a critical role in the lipid metabolic mechanism. Among these gene products, CD36 is a cell surface receptor that binds to a variety of components, including collagen (Tandon et al., 1989), thrombospondin (Silverstein et al., 1992), erythrocytes parasitized with *Plasmodium falciparum* (Oquendo et al., 1989), oxidized low-density lipoprotein (Nicholson et al., 1995), native lipoproteins (Calvo et al., 1998), oxidized phospholipids (Podrez et al., 2002), and long-chain fatty acids (Baillie et al., 1996). Adiponectin (ADIPOQ) is a protein hormone that modulates several metabolic processes, including glucose regulation and fatty acid catabolism. In addition, ADIPOQ is exclusively secreted from adipose tissue into the vasculature and is relatively more abundant compared with other plasma hormones (Diez and Iglesias, 2003). Cyclin D1 (CCND1) has been shown to interact with the tumor suppressor protein retinoblastoma (Rb; Kishimoto et al., 2008) and mutation of its corresponding gene may contribute to tumorigenesis (Barbash et al., 2008). The second most significant network identified in this study was composed of 17 focus genes that encode proteins associated with cellular assembly and organization, gastrointestinal disease, and genetic disorder. In this network, PTGS2 has been reported to be expressed at significantly higher levels in malignant gastrointestinal stromal tumors compared with benign tumors (Miao et al., 2008). The zeta poly peptide chain of CD247 plays a key role in signal transduction after antigen recognition...
by the T-cell receptor, and its downregulation might be responsible for deficient cellular immune responses (Eleftheriadis et al., 2008).

Within the 74 genes that expressed significant alteration after feeding 5 mg/kg of carvacrol, several genes related with the endocrine and metabolic system such as selenoprotein X, 1 (SEPX1) and protease, serine, 3 (PRSS3) were upregulated. Selenoprotein X, 1 (SEPX1) belongs to the methionine sulfoxide reductase B family and has high specificity for reduction of the R forms of free and protein-bound methionine sulfoxide (Moskovitz et al., 2002). Protease, serine, 3 (PRSS3) encodes mesotrypsin in humans and has the property of resistance to biological trypsin inhibitors, such as pancreatic trypsin inhibitor, soybean, lima bean, ovomucoid inhibitor, and α 1-antitrypsin (Rinderknecht et al., 1984). It might be from the biological function of digestive degradation of trypsin inhibitors (Szmola et al., 2003). Carvacrol has well-known antibacterial and antifungal properties as well as antioxidative effects, with several potential applications for topical treatment of mucosal and epithelial infections (Chami et al., 2004a,b; Kristinsson et al., 2005; Burt et al., 2005). The antiinflammatory properties of carvacrol have been suggested to be due to inhibition of inducible PTGS2 isoforms (Landa et al., 2009), a gene we found to be downregulated after dietary supplementation with *Capsicum* oleoresin (Supplemental Table 3; http://ps.fass.org/content/vol89/issue1/).

Among the altered genes after feeding 3 mg/kg of cinnamaldehyde, hemoglobin, β (HBB) was highly upregulated. Hemoglobin, β (HBB) is an oxygen transporter (Thein et al., 1990) and a mutation in HBB causes sickle cell anemia (Persons, 2003).

Generally, the supplementation with carvacrol, cinnamaldehyde, and *Capsicum* had no influence on BW or feed efficiency, but these supplements improved ileal and fecal digestibility in homeostatic status in chickens (McElroy et al., 1994; Hernandez et al., 2004; Jamroz et al., 2005). The detailed mechanisms are not known but may involve morphological modification of cells of the gastrointestinal mucosa (Jamroz et al., 2006) and genetic regulation of metabolic network. Furthermore, challenge of phytomitic-fed birds with *Eimeria* reduced gut lesion, enhanced BW, and decreased fecal oocyst output. In addition, the local production of proinflammatory cytokines was significantly decreased. In our results, several immune-related genes showed changes after treatment with phytomutrients reflecting their well-known medicinal effects against various bacteria or fungi in chicken infection studies (Tellez et al., 1993; Jamroz et al., 2006; Burt et al., 2007). Clearly, these phytomutrients exert significant effects on host immunity, metabolism, and physiology by altering the expression of important genes associated with host disease resistance against pathogens.

In conclusion, transcriptional profiling and pathway analysis revealed differential expression by 3 dietary phytomutrients and identified network of genes induced by *Capsicum* oleoresin. These results provide new information concerning the molecular mechanisms involved in dietary modulation of host immunity, physiology,
and metabolism. Future studies based on these results will contribute to comprehensive understanding of the molecular mechanism of phytonutrients in the chicken digestive tract and will facilitate the development of novel dietary strategies to immunomodulate host response in normal and disease states.

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REFERENCES


