Cinnamaldehyde Enhances in vitro parameters and augments in vivo protection against avian Coccidiosis

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INTRODUCTION

Coccidiosis:
economically important disease of poultry industry
annual economic loss > $ 3 billion world wide.

Increasing governmental regulation of drug use
> Alternative ways to control animal diseases are needed.
Dietary supplementation such as safflower leaf, plum, anethol, mushroom, capsaicin, curcumin, etc enhance immunity in chickens (Lee et al., 2008, 2010a,b, 2011a,b).
Cinnamon treated gastritis and inflammatory diseases.

Cinnamaldehyde (CINN): major constituent of cinnamon, strong antibacterial activity, improved ileal digestibility and gastrointestinal mucosa health.

Hypothesis:
Dietary feeding with CINN may provide an alternative to drug strategy to mitigate intestinal damage caused by avian coccidiosis in poultry.
OBJECTIVES

- To investigate immune enhancing potential of CINN on avian innate immunity and to develop novel immunomodulation strategies to enhance intestinal health in young broiler chickens.

- To investigate the effect of dietary feeding of CINN of young broiler chickens in avian coccidiosis disease challenge model.
< In vitro >
Material

Purified Cinnamaldehyde (CINN) from Pancosma was used after dialysis against PBS for 48 hours.

CINN sample was filtered through a 0.45 μm filter and serially diluted in PBS for in vitro evaluation.

Methods

Splenocyte proliferation and tumor cell growth inhibition were assessed using WST-8 (Cell-Counting Kit-8®).

Nitric oxide production by macrophages was measured using Griess reagent.

Sporozoite viability was measured using trypan blue dye exclusion test.
Fig. 1. Effects of CINN on splenocyte proliferation (2.5x10^6/well, 48 hrs). *** P < 0.001
Fig 2. Effects of CINN on tumor cell growth (1x10^5/well, 48 hrs).

*** $P < 0.001$, * $P < 0.05$
Fig 3. Effects of CINN on NO production (1X10^5/well, 24hrs).

*** P < 0.001
Fig. 4. Anti-parasitic properties of CINN. Numbers on the bars indicate the percentage inhibition against the media control.
<In vivo>
Fig 1. Schematic outline of the experimental design.
<table>
<thead>
<tr>
<th>Analyzed Factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight gain between 0 &amp; 9 days postinfection (dpi)</td>
</tr>
<tr>
<td>Fecal oocysts at 6-9 dpi using McMaster chamber &amp; Lesion score at 9 dpi</td>
</tr>
<tr>
<td>Serum Ab to <em>Eimeria</em> protein at 9 dpi using ELISA</td>
</tr>
<tr>
<td>Cytokine and gene expression in intestine at day 0 using RT-PCR</td>
</tr>
</tbody>
</table>

**Bioinformatic Analysis**
using GeneSpring GX 7.3 & informatics tools
### Table 1. Oligonucleotide primers used for qRT-PCR of chicken cytokines.

<table>
<thead>
<tr>
<th>RNA target</th>
<th>Primer sequences</th>
<th>PCR product size (bp)</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5′-GGTGGTGCTAAGCGTGTTAT-3′</td>
<td>264</td>
<td><strong>K01458</strong></td>
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<tr>
<td>Reverse</td>
<td>5′-ACCTCTGTCATCTCTCCACA-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5′-TGGGCATCAAGGGCTACA-3′</td>
<td>244</td>
<td><strong>Y15006</strong></td>
</tr>
<tr>
<td>Reverse</td>
<td>5′-TCGGGTTGGTTGGTGATG-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5′-CAAGGTGACGGAGGAGGAC-3′</td>
<td>254</td>
<td><strong>AJ309540</strong></td>
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<tr>
<td>Reverse</td>
<td>5′-TGCGGAGGAGGGATTTCT-3′</td>
<td></td>
<td></td>
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<tr>
<td>IL-15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5′-TCTGTTCTTCTGTTCTGAGTGATG-3′</td>
<td>243</td>
<td><strong>AF139097</strong></td>
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<tr>
<td>Reverse</td>
<td>5′-AGTGATTGGCTTCTGTCTTTGGTA-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5′-AGCTGACGGTGACCTATTATT-3′</td>
<td>259</td>
<td><strong>Y07922</strong></td>
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<tr>
<td>Reverse</td>
<td>5′-GGCTTTTGCCTGGATTTC-3′</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2. The composition of 10K AVIELA microarray V2 and array images scanned with Cy3 and Cy5 channels

<table>
<thead>
<tr>
<th>Type of cDNA clones</th>
<th>Number of clones</th>
<th>Number of spots</th>
<th>Detail description</th>
</tr>
</thead>
<tbody>
<tr>
<td>IEL ESTs</td>
<td>9,668</td>
<td>19,336</td>
<td>6,654 genes and 3,014 singleton ESTs</td>
</tr>
<tr>
<td>Controls</td>
<td>6</td>
<td>76</td>
<td>GAPDH, β-actin, soybean genes, and vectors</td>
</tr>
<tr>
<td>Blanks</td>
<td>N/A</td>
<td>494</td>
<td>Spotting solution only</td>
</tr>
<tr>
<td><strong>Total elements</strong></td>
<td><strong>9,674</strong></td>
<td><strong>19,906</strong></td>
<td></td>
</tr>
</tbody>
</table>

+ 474 spot elements from LPS-stimulated macrophages and direct PCR clones of immune-related genes (*Appl Microbiol Biotechnol* 2003;62:392-399)

*Each gene was spotted in duplicates*
Fig 3. Data normalization and analysis

Statistical and bioinformatic analysis using GeneSpring GX 7.3 and informatics tools

Metabolomics analysis using Ingenuity Pathways Analysis
Fig 4. Number of transcripts affected by CINN feeding
Fig 5. Gene ontology analysis for the genes >2 fold up- or down-regulated by CINN

- CPD: Cell proliferation & differentiation
- CPGM: Coenzyme & prosthetic group metabolism
- BG: Blood circulation & gas exchange
- PM: Phosphate metabolism
- CM: Carbohydrate metabolism
- H: Homeostasis
- NNNM: Nucleoside, nucleotide & nucleic acid metabolism
- LFSM: Lipid, fatty acid & steroid metabolism
- AAM: Amino acid metabolism

Intramural protein traffic 3%
Cell cycle 7%
Protein metabolism & modification 13%
Cell structure & motility 7%
Cell proliferation & differentiation 4%
Coenzyme & prosthetic group metabolism 4%
Immunity & defense 3%
Transport 8%
Other metabolism 2%
Apoptosis 3%
Oncogenesis 2%
Developmental processes 4%
Signal transduction 8%
Unknown 6%
Nucleoside, nucleotide & nucleic acid metabolism 12%
<table>
<thead>
<tr>
<th>ID</th>
<th>Molecules in Network</th>
<th>Score</th>
<th>Focus Molecl</th>
<th>Top Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>2-oxoglutaric acid, ADRM1, beta-estradiol, <em>BBB3</em>, <em>DDX1</em>, EGFR, ELF3, <em>FADD</em>, <em>FIR3</em>, <em>FXYD2</em>, HIF1A, hydrogen peroxide, IL6, NRC1, <em>OGDH</em>, PSMA4, PSMA5, <em>PSMA6</em>, PSMA7, PSMB1, PSMB2, PSMB4, PSMB6, PSMB7, PSMC4, PSMD6, RBL1, <em>SFRS7</em>, <em>SIK1</em>, SLC2A4, <em>SLC34A2</em>, <em>STK4</em>, <em>UBE2D1</em>, UCHL5, <em>USP4</em></td>
<td>27</td>
<td>14</td>
<td>Cell Death, Gene Expression, Cellular Development</td>
</tr>
<tr>
<td>5</td>
<td><em>HERC4</em>, HERC6</td>
<td>2</td>
<td>1</td>
<td>Drug Metabolism, Small Molecule Biochemistry, Lipid Metabolism</td>
</tr>
<tr>
<td>6</td>
<td><em>COQ5</em>, methyltransferase</td>
<td>2</td>
<td>1</td>
<td>Drug Metabolism, Small Molecule Biochemistry, Lipid Metabolism</td>
</tr>
<tr>
<td>7</td>
<td>3-hydroxy-3-methylglutaryl-coenzyme A, coenzyme A, hydroxymethylglutaryl-CoA hydrolase, <em>megulitol</em>, succinate-hydroxymethylglutrate CoA-transferase, succinic acid, succinyl-coenzyme A, water</td>
<td>1</td>
<td>1</td>
<td>Drug Metabolism, Small Molecule Biochemistry, Lipid Metabolism</td>
</tr>
</tbody>
</table>
Fig 6. Network

*Top functions:*
Antigen presentation
Humoral Immune response
Inflammatory disease
Fig 7. Effect of CINN on weight gain in EA and EM-infected chickens ($P < 0.05$)
Fig 8. CINN feeding reduced oocyst shedding in EA-infected chickens. ** $P < 0.01$. 
Fig 9. Effect of dietary CINN on serum antibody (EtMIC2) responses following infection with EA, EM, and ET. * P < 0.05, *** P < 0.001.
**Fig 10.** Effect of dietary CINN on intestinal cytokine transcript levels.
**SUMMARY**

*In vitro:*
CINN induced significantly higher splenocyte proliferation, nitric oxide production, inhibition of chicken tumor cell growth, and exerted direct killing effect against *Eimeria* sporozoites.

*In vivo:*
CINN-fed birds showed 10~30% increases in body weight gain and 24~43% decreases in fecal oocysts production compared to the untreated birds following challenge infection with EA, EM or ET. All chickens produced higher IgY antibody titers against coccidia.

The levels of intestinal lymphocyte cytokine transcripts of IL-1β, IL-6, IL-15, or IFN-γ were 2-5 folds higher and several pathways of metabolic and cellular immune response were altered by >2.0 fold in CINN-fed chickens compared to the controls.
Conclusion

• This study provides the first immunological evidence that cinnamaldehyde enhances innate immunity of chickens and increases local protective immunity against avian coccidiosis.

• Therefore, dietary supplementation of young broilers with cinnamaldehyde could be an alternative way to improve gut health and to decrease the use of drugs in poultry production.
Published Papers


Presented at conferences

Lee SH, Lillehoj HS, Jang SI, Kim DK, Jeong MS, Lillehoj EP, Bravo D. Supplementation of phytoneutrients improves immune system and increases resistance to necrotic enteritis in young broiler chickens. PSA annual meeting, Georgia, July 9-12, 2012.


Thank you so much!