Antibiotic alternatives for controlling foodborne pathogens and disease in poultry

Abhi Upadhyay, Ph.D.
Dr. Annie Donoghue, Dr. Narayan Rath, 2 Vacant SY
Poultry Production and Product Safety Research,
Fayetteville, AR

United States Department of Agriculture
Agricultural Research Service
To provide the poultry industry with practical antibiotic alternatives for the control of bacterial foodborne pathogens in conventional and organic poultry sectors.
CRIS project

- Mucin adapted probiotic bacteria
- Mucin adapted bacteriophages
- Campylobacter specific CRISPR-Cas system
- Plant-derived compounds
- Egg-shell Membrane modulator

Project plan

**Pre-harvest control of Campylobacter and Salmonella**
Reduce the incidence of Campylobacter in poultry using
(i) Mucin adapted probiotics
(ii) Plant-derived compounds
(iii) Mucin adapted bacteriophage with or without CRISPR-Cas system
(iv) Egg shell membrane modulator

Reduce the incidence of Salmonella in poultry using
(i) Egg shell membrane modulator

**Post-harvest control of Campylobacter and Salmonella**
Reduce the incidence of Campylobacter and Salmonella on poultry carcass using
(i) Plant-derived compounds
(ii) Mucin adapted bacteriophage with or without CRISPR-Cas system

Investigate the mechanisms of action of probiotic bacteria, bacteriophage and plant-derived compounds for anti-Campylobacter/Salmonella efficacy using phenotypic assay, gene expression analysis and RNA transcriptomics
Plant-derived compounds

• **Major groups:** Polyphenols, Flavonoids, Alkaloids, Lectins, Tannins

• **Advantages:** Multiple mechanisms of action
<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Trans</em>-cinnamaldehyde (TC)</td>
<td>Cinnamon trees</td>
</tr>
<tr>
<td>Carvacrol (CR)</td>
<td>Oregano oil</td>
</tr>
<tr>
<td>Eugenol (EG)</td>
<td>Clove</td>
</tr>
<tr>
<td>β-resorcylic acid (BR)</td>
<td>Resins, Brazilian wood</td>
</tr>
</tbody>
</table>
Strategies to control foodborne pathogens

- **Pre-harvest persistence**
  - Campylobacter spp.
  - Salmonella spp.

- **Persistence in processing environment**
  - Listeria monocytogenes
  - Campylobacter spp.
  - Salmonella spp.

- **Post-harvest contamination**

- **Anti-virulence strategies**
Efficacy of Beta-Resorcylic acid in reducing *Campylobacter jejuni* and *Salmonella* Enteritidis colonization in broiler chicken
Experimental Approach 1

**Plant compound feeding phase**

**Day 0**
- Grouping of birds (Cobb500)
- Control
  - 0.25% BR
  - 0.5% BR
  - 1% BR

**Day 7**
- Orally challenged with *C. jejuni*
  - (7 log CFU/bird)

**Day 14**
- *C. jejuni* enumeration in cecal contents

10 birds/treatment/trial

**Pre-harvest persistence**

**Persistence in processing environment**

**Post-harvest contamination**

**Anti-virulence strategies**
Effect of BR on Campylobacter jejuni cecal colonization


$P<0.05$

Pre-harvest persistence
Persistence in processing environment
Post-harvest contamination
Anti-virulence strategies

~1.5-2 log reduction
Effect of BR on *Campylobacter jejuni* motility and attachment to epithelial cells

Motility

<table>
<thead>
<tr>
<th>Zone of motility (cm)</th>
<th>Control</th>
<th>DMSO</th>
<th>0.0125% BR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><img src="https://example.com/graph1.png" alt="Graph" /></td>
<td><img src="https://example.com/graph2.png" alt="Graph" /></td>
<td><img src="https://example.com/graph3.png" alt="Graph" /></td>
</tr>
</tbody>
</table>

Attachment to epithelial cells

<table>
<thead>
<tr>
<th>Campylobacter counts (Log CFU/ml)</th>
<th>Control</th>
<th>DMSO</th>
<th>0.0125% BR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><img src="https://example.com/graph4.png" alt="Graph" /></td>
<td><img src="https://example.com/graph5.png" alt="Graph" /></td>
<td><img src="https://example.com/graph6.png" alt="Graph" /></td>
</tr>
</tbody>
</table>

P<0.05

Pre-harvest persistence

Persistence in processing environment

Post-harvest contamination

Anti-virulence strategies
Effect of BR on *C. jejuni* chicken colonization genes

- Pre-harvest persistence
- Persistence in processing environment
- Post-harvest contamination
- Anti-virulence strategies

**Figure:**

Bar chart comparing the effect of BR on *C. jejuni* colonization genes. The chart shows the relative quantification of genes involved in motility and attachment.

- **Motility** genes: *motA*, *motB*, *fliA*
- **Attachment** genes: *ciaB*, *jlpA*

Significance level: *P* < 0.05
Experimental Approach 2

**Plant compound feeding phase**

**Day 0**
Grouping of birds (Cobb500)

**Control**
- 0.5% BR
- 1% BR

**Day 7**
Orally challenged with S. Enteritidis (7 log CFU/bird)

**Day 21**
S. Enteritidis enumeration in cecum liver crop

- 10 birds/treatment/trial

**Pre-harvest persistence**
**Persistence in processing environment**
**Post-harvest contamination**
**Anti-virulence strategies**
Effect of BR on *S. Enteritidis* cecal, crop, and liver colonization in 21 day old broiler chickens

Cecum

Liver

Crop

S. Enteritidis (Log CFU/ml)

Control 1% BR 0.5% BR

Cecum:
- ~2-3 log reduction

Liver:
- Control: a
- 1% BR: b
- 0.5% BR: b

Crop:
- Control: a
- 1% BR: b
- 0.5% BR: b
Strategies to control foodborne pathogens (1)

Pre-harvest persistence

Persistence in processing environment

Post-harvest contamination

Anti-virulence strategies

C. jejuni
Bacterial biofilms

Pre-harvest persistence

Persistence in processing environment

Post-harvest contamination

Anti-virulence strategies

Micro-colonies

Mature biofilm

Food products
Campylobacter and Salmonella biofilms

Campylobacter

Salmonella spp.

Brown et al., 2013. Journal of Applied Microbiology, 115, 1212-1221


Pre-harvest persistence

Persistence in processing environment

Post-harvest contamination

Anti-virulence strategies
Antibiofilm strategy

Inhibition of biofilm formation

Inactivation of mature biofilm

Pre-harvest persistence
Persistence in processing environment
Post-harvest contamination
Anti-virulence strategies
The efficacy of phytochemicals in controlling *C. jejuni* biofilms
Inhibition of *C. jejuni* biofilm formation by phytochemicals

Phytochemicals +
*C. jejuni*  
(≈6 log CFU)

20°C for 3 days

Quantification of *C. jejuni* biofilm  
(TTC staining)  
(24, 48, 72 h)

Brown et al., 2013, J Appl Microbiol; Brown et al., 2014 AEM
Inhibition of *C. jejuni* biofilm formation by phytochemicals

**Absorbance at 500nm**

<table>
<thead>
<tr>
<th>Time in hours</th>
<th>Control</th>
<th>TC</th>
<th>Eugenol</th>
<th>Carvacrol</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td><img src="24h_control" alt="Absorbance" /></td>
<td><img src="24h_TC" alt="Absorbance" /></td>
<td><img src="24h_Eugenol" alt="Absorbance" /></td>
<td><img src="24h_Carvacrol" alt="Absorbance" /></td>
</tr>
<tr>
<td>48 h</td>
<td><img src="48h_control" alt="Absorbance" /></td>
<td><img src="48h_TC" alt="Absorbance" /></td>
<td><img src="48h_Eugenol" alt="Absorbance" /></td>
<td><img src="48h_Carvacrol" alt="Absorbance" /></td>
</tr>
<tr>
<td>72 h</td>
<td><img src="72h_control" alt="Absorbance" /></td>
<td><img src="72h_TC" alt="Absorbance" /></td>
<td><img src="72h_Eugenol" alt="Absorbance" /></td>
<td><img src="72h_Carvacrol" alt="Absorbance" /></td>
</tr>
</tbody>
</table>

P<0.05

**Pre-harvest persistence**

**Persistence in processing environment**

**Post-harvest contamination**

**Anti-virulence strategies**
Inactivation of *C. jejuni* biofilm by phytochemicals

Phytochemicals
20°C for 1, 5, 10 min

*C. jejuni* (≈6 log CFU)

24 h

Enumeration of *C. jejuni* in biofilm

Pre-harvest persistence

Persistence in processing environment

Post-harvest contamination

Anti-virulence strategies
Inactivation of mature biofilm by phytochemicals

Persistence in processing environment

Anti-virulence strategies

Pre-harvest persistence

Post-harvest contamination

~ 4 log reduction

Mature biofilm

~ 6 log reduction

Control

Campylobacter jejuni counts (Log CFU/ml)

Time in minutes

TC 0.25%

TC 0.5%

TC 1%

0 2 4 6 8 10 12
Strategies to control foodborne pathogens (2)

Pre-harvest persistence

Persistence in processing environment

Post-harvest contamination

Anti-virulence strategies
Efficacy of Beta-resorcylic acid in reducing *C. jejuni* on chicken skin and meat.

CONTAMINATION OF CHICKEN CARCASS

Contamination during slaughter

- Pre-harvest persistence
- Persistence in processing environment
- Post-harvest contamination
- Anti-virulence strategies
Inactivation of *C. jejuni* on chicken skin and meat by Beta-resorcylic acid

*C. jejuni* inoculation
(≈6 log CFU/sample)

Antimicrobial wash at 25°C
30 sec

Enumeration of surviving *C. jejuni*

---

Pre-harvest persistence

Persistence in processing environment

Post-harvest contamination

Anti-virulence strategies
Effect of Beta-resorcylic acid on survival of *C. jejuni* on chicken skin

![Graph showing the effect of Beta-resorcylic acid on survival of *C. jejuni* on chicken skin.](image)

- **Pre-harvest persistence**
- **Persistence in processing environment**
- **Post-harvest contamination**
- **Anti-virulence strategies**
Effect of Beta-resorcylic acid on survival of *C. jejuni* on chicken breast meat

![Graph showing the effect of Beta-resorcylic acid on *C. jejuni* survival in chicken breast meat.](image)

- **Trial 1**: Pre-harvest persistence and persistence in processing environment.
- **Trial 2**: Post-harvest contamination and anti-virulence strategies.

- **Control**
- **0.5% BR**
- **1% BR**
- **2% BR**
Strategies to control foodborne pathogens (3)

- Pre-harvest persistence
- Persistence in processing environment
- Post-harvest contamination
- Anti-virulence strategies
Virulence factors in *C. jejuni*

Croinin and Backert, 2012
Efficacy of phytochemicals in reducing *Campylobacter jejuni* virulence attributes *in vitro*

Strains- ATCC-11168, ATCC 81-176, Wild type S-8
<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Trans</em>-cinnamaldehyde (TC)</td>
<td>Cinnamon trees</td>
</tr>
<tr>
<td>Carvacrol (CR)</td>
<td>Oregano oil</td>
</tr>
<tr>
<td>Eugenol (EG)</td>
<td>Clove</td>
</tr>
</tbody>
</table>

- **Pre-harvest persistence**
- **Persistence in processing environment**
- **Post-harvest contamination**
- **Anti-virulence strategies**
Sub-inhibitory concentrations

- Compound concentrations not inhibiting bacterial growth but modulating virulence

(Zhanel et al., 1992; Fonseca et al., 2004; Johny et al., 2010; Upadhyay et al., 2012)

**Hypothesis:** Phytochemicals (TC, CR, EG) reduce the expression of critical virulence factors in *Campylobacter jejuni*
## Phytochemical concentrations

<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>SICs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Trans</em>-cinnamaldehyde (TC)</td>
<td>0.005</td>
</tr>
<tr>
<td>Carvacrol (CR)</td>
<td>0.001</td>
</tr>
<tr>
<td>Eugenol (EG)</td>
<td>0.005</td>
</tr>
</tbody>
</table>

- **Pre-harvest persistence**
- **Persistence in processing environment**
- **Post-harvest contamination**
- **Anti-virulence strategies**
Specific objectives

To investigate the efficacy of TC, CR, EG on *C. jejuni*

- Motility
- Adhesion to and invasion of epithelial cells
- Translocation of epithelial barrier
- Production of CDT
- Expression of virulence genes
Effect of phytochemicals on *C. jejuni* motility

Motility agar (0.3%)

5 µl *C. jejuni* (≈6 log CFU)

Microaerophilic Incubation (37°C for 24 h)

(Niu and Gilbert, 2004)

- Pre-harvest persistence
- Persistence in processing environment
- Post-harvest contamination
- Anti-virulence strategies
Effect of phytochemicals on *C. jejuni* motility

Human body temperature

- Control
- Ethanol
- TC 0.005
- TC 0.01
- CR 0.001
- CR 0.002
- EG 0.005
- EG 0.01

**Treatments**

<table>
<thead>
<tr>
<th>Zone of motility (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
</tr>
<tr>
<td>a</td>
</tr>
<tr>
<td>e</td>
</tr>
<tr>
<td>e</td>
</tr>
<tr>
<td>b</td>
</tr>
<tr>
<td>c</td>
</tr>
<tr>
<td>c</td>
</tr>
<tr>
<td>d</td>
</tr>
</tbody>
</table>

Chicken body temperature

- Control
- Ethanol
- TC 0.01%
- CR 0.002%
- EG 0.01%

**Treatments**

<table>
<thead>
<tr>
<th>Zone of motility (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
</tr>
<tr>
<td>a</td>
</tr>
<tr>
<td>d</td>
</tr>
<tr>
<td>b</td>
</tr>
<tr>
<td>c</td>
</tr>
</tbody>
</table>

**P<0.05**

Pre-harvest persistence

Persistence in processing environment

Post-harvest contamination

Anti-virulence strategies
Attachment, invasion and translocation of epithelial barrier by *C. jejuni*

Effect of phytochemicals on *C. jejuni* adhesion/invasion to intestinal cells

*Campylobacter jejuni*  

Phytochemicals

Caco-2/Chicken primary enterocytes

10^6 *C. jejuni*

Control  

With phytochemicals

MOI 1:10

Incubation (37°C, 1.5 h under 5% CO₂)  

Bacterial enumeration

Moroni et al. 2006

Pre-harvest persistence  

Persistence in processing environment  

Post-harvest contamination  

Anti-virulence strategies
Effect of phytochemicals on *C. jejuni* adhesion to intestinal cells

Human intestinal cells (Caco-2)

- Control
- Ethanol
- TC 0.005
- TC 0.01
- CR 0.001
- CR 0.002
- EG 0.005
- EG 0.01

Chicken primary epithelial cells

- Control
- Ethanol
- TC 0.01%
- CR 0.002%
- EG 0.01%

Pre-harvest persistence
Persistence in processing environment
Post-harvest contamination
Anti-virulence strategies

P<0.05

~1-1.5 log reduction
Effect of phytochemicals on *C. jejuni* invasion of intestinal cells* (1)

![Graph showing the effect of phytochemicals on C. jejuni invasion](image)

*P<0.05

<table>
<thead>
<tr>
<th>Pre-harvest persistence</th>
<th>Persistence in processing environment</th>
<th>Post-harvest contamination</th>
<th>Anti-virulence strategies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Ethanol</td>
<td>TC 0.005</td>
<td>TC 0.01</td>
</tr>
<tr>
<td>CR 0.001</td>
<td>CR 0.002</td>
<td>EG 0.005</td>
<td>EG 0.01</td>
</tr>
</tbody>
</table>
Effect of phytochemicals on *C. jejuni* translocation of intestinal cells (2)

10^6 C. jejuni

Caco-2 on membrane filter (5 um)

Incubation (37°C 3h under 5% CO₂)

Bacterial enumeration

Koo, Bhunia et al., 2012. PloS One
Effect of phytochemicals on *C. jejuni* translocation of intestinal cells (3)

![Graph showing the effect of different treatments on the number of *C. jejuni* translocated.](image)

- **Pre-harvest persistence**
- **Persistence in processing environment**
- **Post-harvest contamination**
- **Anti-virulence strategies**

P < 0.05
C. jejuni Cytolethal distending toxin

Extracellular space

Cytoplasm

Nucleus

Effect of phytochemicals on *C. jejuni* CDT production (1)

*C. jejuni* (37°C for 24 h) With or without phytochemicals

Supernatant (CDT) collected

Incubation 9 days, 37°C

CDT quantification

LDH based fluorescence assay

Caco-2 cells

Pre-harvest persistence

Persistence in processing environment

Post-harvest contamination

Anti-virulence strategies

LDH

Lactate → Pyruvate

NAD⁺ → NADH

Resorufin

Diaphorase

Resazurin
Effect of phytochemicals on *C. jejuni* CDT production (2)

![Graph showing the effect of phytochemicals on CDT production](image)

- **Control**
- **Ethanol**
- **TC 0.005%**
- **TC 0.01%**
- **CR 0.001**
- **CR 0.002%**
- **EG 0.005%**
- **EG 0.01%**

**Treatments**

**Percentage cytotoxicity**

- **P<0.05**

**Legend:**

- a
- b
- c
- bc

**Legend:**

- Pre-harvest persistence
- Persistence in processing environment
- Post-harvest contamination
- Anti-virulence strategies
Effect of trans-cinnamaldehyde on expression of C. jejuni virulence genes*

Trans-cinnamaldehyde 0.01%

*Treatments significantly different from the control at P<0.05
Effect of carvacrol and eugenol on expression of *C. jejuni* virulence genes*

**Carvacrol 0.002%**

**Eugenol 0.01%**

*Treatments significantly different from the control at P<0.05*
**Key takeaways...**

Phytochemicals reduced *C. jejuni* and *S. Enteritidis* colonization

Phytochemicals reduced *C. jejuni* biofilms on food contact surface

Phytochemicals reduced *C. jejuni* survival on chicken skin and meat

Phytochemicals modulate *C. jejuni* virulence properties
Future research

Effect of phytochemicals on *C. jejuni* transcriptome and cecal microbiome of chickens

Developing novel anti-biofilms strategies for controlling *C. jejuni*

Effect of phytochemical nanoemulsion in reducing *C. jejuni* on poultry products

Developing anti-virulence strategies for controlling *C. jejuni* colonization in chickens and infection in humans
Acknowledgments

Dr. Annie M. Donoghue (USDA-ARS)
Dr. Narayan Rath (USDA-ARS)
Dr. Dan J. Donoghue (University of Arkansas)
Dr. Kumar Venkitanarayanan (University of Connecticut)

Our team
Dr. Pamela J. Blore, Dr. Komala Arsi, Dr. Indu Upadhyaya
Sandip Shrestha, MS; Basanta Wagle, MS; Sarah Wright, MS

Funding
OREI program
Thank You

“Let food be thy medicine and medicine be thy food”

Hippocrates
Salmonella colonization in chicken

Haraga et al., 2008; Nature Reviews Microbiology
Campylobacter colonization in chicken

Young et al., 2007
Nature Reviews Microbiology