Effects of Trans-Cinnamaldehyde on Campylobacter and Sperm Viability in Chicken Semen after In vitro Storage†

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Abstract: Campylobacter is one of the leading causes of bacterial human acute gastroenteritis. These microorganisms are highly prevalent in poultry semen and may contribute to vertical transmission of the pathogen between the breeder hen and offspring. Unfortunately, strategies to reduce or eliminate these pathogens in poultry semen negatively impact sperm viability. Many plant essential oils have been reported to exhibit antimicrobial activity against bacteria, fungi and viruses. The objective of our study was to examine the efficacy of trans-cinnamaldehyde, the main component in cinnamon oil, to reduce Campylobacter concentrations in chicken semen. Semen was collected from roosters, pooled and diluted with semen extender, then divided into treatments: negative control (no Campylobacter, no trans-cinnamaldehyde), positive control (inoculated with Campylobacter, no trans-cinnamaldehyde) or treatments containing concentrations of 0.24, 0.12, 0.06, 0.03 or 0.015% trans-cinnamaldehyde. Treatment groups receiving Campylobacter were then immediately inoculated with ~105 cfu/mL of a wild-type Campylobacter jejuni and held at 4°C or 23°C for 2 h. Semen was stored at 4°C for an additional 24 h and assessed for Campylobacter concentrations and sperm viability at 2, 6 and 24 h utilizing SYBR 14/Propidium iodide live/dead stain and fluorescent microscopy. The study was replicated eight times. After 2h at 23°C a 2 log reduction in Campylobacter counts were observed in the 0.12 and 0.24% trans-cinnamaldehyde treatment groups compared to positive controls. In the 4°C treatments, no differences were observed between treatments and controls after 2 h. Samples evaluated after 24 h incubation in vitro at 4°C, showed significant reductions of Campylobacter counts in the 0.06, 0.03 or 0.015% trans-cinnamaldehyde treatments groups, while the 0.12 and 0.24% groups eliminated detectable Campylobacter counts. Sperm viability remained at 80% or above for all treatment groups. Trans-cinnamaldehyde reduced Campylobacter in semen, without detrimentally affecting sperm viability and might provide a practical solution to eliminate Campylobacter in poultry semen after in vitro storage.

Key words: Campylobacter, trans-cinnamaldehyde, chicken, sperm

INTRODUCTION
Campylobacter, a Gram-negative bacterium, is one of the leading bacterial causes of human acute gastroenteritis in many countries, especially in developed countries (Centers for Disease Control and Prevention, 2007; Workman et al., 2006). Campylobacter is commonly found in the gastrointestinal tract of most wild and domestic animals (Wagenaar et al., 2006), however, epidemiological evidence indicates that consumption or handling of raw or undercooked contaminated poultry products are the most common sources of human Campylobacter infections (Corry and Attabay, 2001; Lee and Newell, 2006; Wong et al., 2007). Due to the ubiquitous nature of the organism, there are multiple potential sources for flock infection, leading to 75-90% Campylobacter prevalence among poultry flocks in the United States (Stern et al., 2001). Numerous studies have also documented the incidence and prevalence of this pathogen in the reproductive tract of both male and female poultry (Buhr et al., 2002; Hiett et al., 2002a, 2002b, 2005a, 2005b; Cole et al., 2004, 2006), in poultry semen (Hiett et al., 2003; Donoghue et al., 2004; Buhr et al., 2005; Cole et al., 2002a, 2002b, 2005a; Cole et al., 2006, Vizzier-Thaxton et al., 2006), hatcheries and eggs (Hiett et al., 2002b; Byrd et al., 2007) indicating that vertical transmission of the organism (from breeders to their offspring) can play an important role in the transmission and incidence of Campylobacter in poultry flocks (Cox et al., 2002c; Wagenaar et al., 2006). Unfortunately, studies have shown that strategies such as addition of antibiotics or iron chelators to conventional poultry semen diluents, or
changes in oxygen or temperatures conditions during storage, have not been completely effective in the reduction or elimination of Campylobacter from poultry semen (Cole et al., 2004, 2006; Donoghue et al., 2004). A potential strategy to control Campylobacter in semen is use of the active components of plant-derived essential oils. Trans-cinnamaldehyde is a natural compound that is the principal component in cinnamon oil (Cinnamomum verum). Trans-cinnamaldehyde has been reported to possess antimicrobial activity towards a wide range of pathogens, including Clostridium botulinum (Bowles and Miller, 1993), Clostridium perfringens (Si et al., 2009), Staphylococcus aureus (Bowles et al., 1995), E. coli O157:H7, Campylobacter jejuni, Listeria monocytogenes and Salmonella enterica (Friedman et al., 2002; Kollanoor Johny et al., 2008, 2010). Recently it was demonstrated that trans-cinnamaldehyde was effective in reducing Salmonella Enteritidis and Campylobacter jejuni in chicken cecal contents and could potentially be used to control these pathogens in chickens through the drinking water on farms (Kollanoor Johny et al., 2008; 2010). Thus, the objective of the present study was to evaluate the potential efficacy of trans-cinnamaldehyde in reducing Campylobacter concentrations from chicken semen after in vitro storage.

MATERIALS AND METHODS

Oil preparation: Trans-cinnamaldehyde (Sigma-Aldrich, St. Louis, MO) was dissolved (0.48%, vol:vol) in Field Ready Green Extender (IMV International Corp, North Maple Grove, MN) with 30% 2-hydroxypropyl-$
\text{-}$\text{cyclodextrin (Sigma-Aldrich, St. Louis, MO)} before adding to chicken semen. Concentrations of trans-cinnamaldehyde were selected based on its effect on chicken sperm viability (data not shown) and its activity against Campylobacter jejuni (Friedman et al., 2002).

Sample preparation: Thirty 48 week old roosters were individually caged, fed standard diets ad libitum and kept under a 14L: 10D photoperiod during the experiment. The study was replicated 8 times. In each trial, semen samples from the roosters were collected by abdominal massage and aspirated into sterile test tubes. Pooled semen were diluted 1:1 (vol:vol) with Field Ready Green semen extender. Then, 400 ul semen aliquoted into 500 ul control or treatments containing 0.24, 0.12, 0.06, 0.03 or 0.015% trans-cinnamaldehyde (diluted in Green Extender). The negative control (no Campylobacter or the positive control (inoculated with Campylobacter) groups received no trans-cinnamaldehyde. Each treatment group, except for the negative control, was then inoculated with 10$^5$ cfu/ml of a wild-type C. jejuni isolate (previously collected from chicken semen) in 100 ul of Campylobacter Enrichment Broth (CEB). The negative control received 100 ul of CEB alone. Conventionally, poultry semen is collected and inseminated within a short period and thus it is kept at room temperature (23°C). Alternatively, if a longer period will occur before insemination, the semen is kept at refrigeration temperature (4°C). To simulate both possibilities, samples were evaluated at room temperature (23°C) after 2 h or at 4°C for 2, 6 and 24 h with agitation (150 rpm; Thurston et al., 1998).

Enumeration of bacteria and sperm viability: After each in vitro storage interval, a sample was taken from each treatment group, serially diluted with Butterfield’s phosphate diluent (BPD, 1:10) and plated on duplicate Campy Line agar plates for enumeration (CLA: Line, 2001). The plates were then incubated for 48 h at 42°C in a microaerophilic environment (5% O$_2$, 10% CO$_2$ and 85% N$_2$). After incubation, characteristic colonies were confirmed as Campylobacter by observation of typical cellular morphology using a phase contrast microscope and with a commercial latex agglutination test kit (Pan Bio Inc., Columbia, MD) specific for C. jejuni, C. coli and C. lariis. The colonies on each CLA plate were counted on a Leica Darkfield plate colony counter (Leica Inc., Buffalo, NY) and the direct counts were converted to log$_{10}$ colony-forming units per milliliter of extender. At each time point, sperm viability was assessed for each treatment group utilizing SYBR 14/Propidium iodide live/dead kit and fluorescent microscopy according to the method of Donoghue et al. (1995).

**Statistical analysis:** Data were analyzed using the GLM procedure of SAS (SAS Institute, 2002). The number of Campylobacter colonies was logarithmically transformed (log$_{10}$ cfu/ml) before analysis to achieve homogeneity of variance. Sperm viability data expressed as percentages were arc sine transformed before analysis. A probability of p<0.05 was required for statistical significance. The data in Table 1 and 2 are shown as arithmetic means for clarity of presentation.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Treatment</th>
<th>2 h</th>
<th>6 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>23°C</td>
<td>Negative control</td>
<td>95$^a$</td>
<td>95$^a$</td>
<td>95$^a$</td>
</tr>
<tr>
<td></td>
<td>Positive control</td>
<td>93$^a$</td>
<td>95$^a$</td>
<td>94$^b$</td>
</tr>
<tr>
<td></td>
<td>Trans-cinnamaldehyde (0.24%)</td>
<td>84$^c$</td>
<td>93$^a$</td>
<td>88$^a$</td>
</tr>
<tr>
<td></td>
<td>Trans-cinnamaldehyde (0.12%)</td>
<td>92$^a$</td>
<td>95$^a$</td>
<td>91$^b$</td>
</tr>
<tr>
<td></td>
<td>Trans-cinnamaldehyde (0.06%)</td>
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<td>95$^a$</td>
<td>94$^a$</td>
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<tr>
<td></td>
<td>Trans-cinnamaldehyde (0.03%)</td>
<td>94$^a$</td>
<td>95$^a$</td>
<td>94$^a$</td>
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<tr>
<td></td>
<td>Trans-cinnamaldehyde (0.015%)</td>
<td>94$^a$</td>
<td>95$^a$</td>
<td>94$^a$</td>
</tr>
</tbody>
</table>

$^a$Means with no common superscript within columns differ significantly (p<0.05).
$^b$All data were arc sine transformed for statistical analysis. For clarity of presentation, arithmetic means are presented.
$^c$In 8 separate trials, sperm viability of each treatment was assessed utilizing SYBR 14/Propidium iodide live/dead kit.
RESULTS
The effect of trans-cinnamaldehyde on sperm viability is depicted in Table 1. At 23°C, the 0.24% trans-cinnamaldehyde group significantly reduced sperm viability after 2 hrs storage, but the value was still above 80% which would be acceptable for artificial insemination. The other trans-cinnamaldehyde groups maintained sperm viability equal to the positive and negative controls, suggesting that trans-cinnamaldehyde at these concentrations does not negatively affect chicken spermatozoa when stored for 2 h at 23°C. At 4°C, all trans-cinnamaldehyde groups maintained sperm viability equal to the controls at 2 h of storage (Table 1). After 6 h of storage at 4°C, trans-cinnamaldehyde at 0.12% and 0.24% sperm viability was reduced but still maintained 88-91% viability whereas treatments of 0.015-0.06% were not different from control (Table 1). Sperm viability in all the trans-cinnamaldehyde groups and the positive control were lower than that of the negative control at 24 h of storage at 4°C, however, sperm viability ranged from 80% to 91%, which would still be acceptable for artificial insemination.

The effect of trans-cinnamaldehyde on Campylobacter in chicken semen is depicted in Table 2. Trans-cinnamaldehyde lowered Campylobacter counts at 2 h 23°C in the 0.12% and 0.24% groups by 2 logCFU/ml. At 2 h of storage at 4°C trans-cinnamaldehyde had no effect on reducing Campylobacter. However, by 6 h of storage at 4°C, Campylobacter was reduced in the 0.12-0.24% groups compared to control. Campylobacter was not detected in the 0.12% and 0.24% trans-cinnamaldehyde groups at 24 h 4°C and all other treatments were significantly lower than the control.

DISCUSSION
Campylobacter is one of the leading bacterial causes of human foodborne infections in the United States (Friedman et al., 2000; Centers for Disease Control and Prevention, 2007). Epidemiological evidence has emphasized the importance of poultry products as a significant source of human Campylobacter infection (Jacobs-Reitsma, 2000; Corry and Attabay, 2001). Studies suggest that the organism is highly prevalent in poultry semen and may contribute to vertical transmission between the breeder hen and offspring (Cox et al., 2002b; Cole et al., 2004, 2006). Unfortunately, strategies to reduce or eliminate Campylobacter in poultry semen, such as aeration, reduced storage temperatures and dilution with extenders containing antibiotics or iron chelators have not been completely effective because they either did not reduce Campylobacter levels or negatively impacted sperm viability parameters (Cole et al., 2004; 2006; Donoghue et al., 2004).

Trans-cinnamaldehyde, a major component of the bark extract of cinnamon, is a food-grade chemical approved by the Food and Drug Administration as Generally Regarded as Safe (21 CFR 182.60). Trans-cinnamaldehyde has reported antimicrobial activity towards a wide range of foodborne pathogens, including Gram-positive and Gram-negative bacteria (Bowles and Miller, 1993; Bowles et al., 1995; Friedman et al., 2002). Friedman and co-workers (2004) demonstrated that trans-cinnamaldehyde can reduce pathogenic bacteria at refrigeration temperatures, which would suggest its potential for reducing Campylobacter in poultry semen kept temporarily under refrigeration temperatures for insemination practices.

The data obtained in this study indicated that trans-cinnamaldehyde has the potential as an antimicrobial agent for reducing Campylobacter in chicken semen. At 0.12% and 0.24% trans-cinnamaldehyde after 24 h of in vitro storage, Campylobacter was not detectable yet acceptable sperm viability was maintained. Follow up studies to determine the influence of trans-cinnamaldehyde on fertility are needed, however, these studies show that trans-cinnamaldehyde can effectively reduce Campylobacter.
reduce *Campylobacter* from semen without severely impacting sperm viability. Trans-cinnamaldehyde could be used to reduce this pathogen in semen used for artificial insemination and to further reduce vertical transmission of *Campylobacter* in chicken flocks.

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**REFERENCES**


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