

## Research

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# Chitosan Supplementation Reduces Enteric Colonization of *Campylobacter jejuni* in Broiler Chickens and Down-Regulates Expression of Colonization Genes

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### ABSTRACT

*Campylobacter* is one of the leading causes of foodborne bacterial gastroenteritis worldwide, and poultry is considered as the most common source of human infections. *Campylobacter* is prevalent in most poultry flocks and a reduction of *Campylobacter* in poultry would greatly reduce the risk of campylobacteriosis in humans. Unfortunately, efforts to reduce *Campylobacter* in pre-harvest poultry have been met with limited success. Preliminary studies with the natural compound, chitosan, demonstrate its ability to kill *Campylobacter*, *in vitro*. The purpose of this study was to determine the ability of feed supplemented chitosan to reduce enteric *Campylobacter* colonization in broiler chickens. Additionally, the effect of chitosan on expression of *Campylobacter*'s chicken colonization genes was investigated using real-time quantitative PCR (RT-qPCR). Because chitosan's antimicrobial properties may vary depending upon its molecular weight, selected doses of three molecular weight chitosans were supplemented in the feed and evaluated for efficacy to reduce *Campylobacter* in chickens. Three replicate trials were conducted, and in each trial, birds were divided into 10 treatments (n=10 birds/treatment) and were fed 0% (controls), 0.25%, 0.5% or 1% (wt./wt.) of a low, medium or high molecular weight chitosan. Birds were fed treated feed for the duration of the study and orally challenged with a four-strain mixture of wild type *C. jejuni* on day 6. On day 15, the ceca samples were collected for enumeration of *Campylobacter*. In all three trials, the 0.5% dose of the medium molecular weight chitosan reduced cecal *Campylobacter* counts (P<0.05). RT-qPCR analysis revealed that chitosan down-regulated the expression of chicken colonization genes as compared to control (P<0.05). These results suggest that supplementation of chitosan in feed is a potential strategy to reduce the enteric colonization of *Campylobacter* in pre-harvest chickens.

**KEYWORDS:** *Campylobacter jejuni*; Chitosan; Broiler chickens; Pre-harvest; Colonization gene; Real-time quantitative PCR.

**ABBREVIATIONS:** RT-qPCR: Real-time quantitative PCR; IBS: Irritable Bowel Syndrome; ReA: Reactive arthritis; IBD: Inflammatory Bowel Disease; BPD: Butterfield's Phosphate Diluent; NCBI: National Center for Biotechnology Information; LMW: Low Molecular Weight; MMW: Medium Molecular Weight; HMW: High Molecular Weight; ECDC: European Centre for Disease Prevention and Control; SIC: Sub-Inhibitory Concentration.

## INTRODUCTION

*Campylobacter* is one of the most frequently reported food-borne pathogens and causes an estimated 1.3 million infections in the United States annually.<sup>1</sup> While the majority of *Campylobacter* cases result in acute gastroenteritis, infection has also been associated with more severe diseases, including Guillain-Barré syndrome, Reactive arthritis (ReA), Irritable Bowel Syndrome (IBS), and Inflammatory Bowel Disease (IBD).<sup>2</sup> Epidemiological evidence indicates that the most common source for *Campylobacter* infections in humans is due to consumption of poultry products.<sup>3</sup> This is typically caused by the consumption of improperly cooked chicken or cross-contamination from handling raw chicken.<sup>1,4</sup> *Campylobacter* colonization in poultry is common; as many as 90% of US broiler flocks are contaminated with this food-borne pathogen.<sup>5</sup> Therefore, a reduction or elimination of *Campylobacter* in poultry is a research priority to reduce the risk of infection in humans. Many pre-harvest strategies have been evaluated for reducing *Campylobacter* in poultry, such as bio-security, probiotics, competitive exclusion, bacteriocins, bacteriophages, vaccines, and natural compounds, often with limited success.<sup>6</sup> Recently, the natural product chitosan has shown potential to reduce colonization of another food-borne pathogen, *Salmonella* Typhimurium, in pre-harvest poultry<sup>7</sup> and may have application against *Campylobacter*. Chitosan has also shown efficacy against other Gram-negative species, including *Escherichia coli* and *Pseudomonas fluorescens*.<sup>8,9</sup> Chitosan, a natural by-product derived from the deacetylation of chitin, is obtained from crab and shrimp shell waste.<sup>10,11</sup> Chitosan is a potential natural food preservative with broad antimicrobial benefits.<sup>8,12,13</sup> Although the exact mode of action of chitosan is not completely understood, researchers have previously determined that chitosan is capable of interacting with the outer cell membrane of bacterial pathogens, altering its permeability, disrupting cellular physiology and causing cell death.<sup>9,14</sup> To our knowledge, the ability of chitosan to reduce *Campylobacter* colonization in poultry has not been evaluated. The purpose of this study was to determine the efficacy of in-feed supplementation of chitosan on *Campylobacter* colonization in broiler chicks. Young chickens were used in this study because previous results from our laboratory demonstrated that young birds can be used as a reliable model to study *Campylobacter* colonization in market age birds.<sup>15,16</sup>

## MATERIALS AND METHODS

### Chitosan Materials

Chitosan of molecular weight 50-190 kDa and 190-310 kDa was obtained from Sigma-Aldrich (St. Louis, MO, USA), and 400-600 kDa chitosan was purchased from Spectrum Chemicals (New Brunswick, NJ, USA).

### In vitro Susceptibility of *C. jejuni* to Chitosan

Antimicrobial activity of each molecular weight chi-

tosan, low (50-190 kDa), medium (190-310 kDa) and high (400-600 kDa), in a 0.5% (wt./vol.) solution was determined by inoculating each solution with a four-strain mixture of wild-type *C. jejuni*. Preparation of the *Campylobacter* inoculum was done as described previously by Farnell and others.<sup>17</sup> In brief, working stock cultures of the four wild-type strains of *C. jejuni* were obtained by individually inoculating each strain into fresh *Campylobacter* Enrichment Broth (CEB, Acumedia, Neogen Corporation, Lansing, MI, USA) from frozen glycerol stock and successively sub-culturing twice at 42 °C for 48 h under microaerophilic conditions. Strain mixtures were then combined centrifuged at 3000 \* g for 10 minutes and the cell pellet re-suspended in 10 mL Butterfield's Phosphate Diluent (BPD). A 1% stock solution (wt./vol.) of each molecular weight of chitosan was prepared in 50 mM acetic acid as described by Ganan and others.<sup>18</sup> For the experiment, the stock concentration of each of the chitosan solutions and the acetic acid control was diluted 1:1 with an inoculum containing 10<sup>8</sup> CFU/mL of *C. jejuni*, resulting in a final concentration of 0.5% for each chitosan. Sample time points included 0, 2, 4 and 8 h post inoculation. At each time point, an aliquot from the treatments and control was taken and 1:10 serial dilutions were direct plated on Campy Line Agar.<sup>19</sup> The plates were incubated for 48 h at 42 °C in a microaerophilic atmosphere. Direct enumeration of *Campylobacter* colonies was converted to CFU/mL for each treatment. Each susceptibility assay was repeated in duplicate.

### In vivo Susceptibility of *C. jejuni* to Chitosan

Day of hatch Cobb broiler chicks (Siloam Springs, AR, USA) from a local commercial hatchery were utilized for the animal experiments. In each of three replicate trials, 100 chicks per trial were randomly divided into 10 treatments, which consisted of three concentrations (0.25%, 0.5%, or 1% wt./wt.) of each molecular weight chitosan, which was added to the feed and a positive control (0% chitosan). Birds were placed in floor pens and provided feed and water *ad libitum*; treated feed was provided throughout the entire trial.

The *Campylobacter* challenge was prepared as mentioned above. Birds were challenged by oral gavage with 0.25 mL of a four-strain mixture of wild-type *C. jejuni* on day 6, at a concentration of 10<sup>7</sup>-10<sup>8</sup> CFU/mL. On day 15, birds were euthanized and the ceca were excised for *Campylobacter* enumeration. Cecal contents were serially diluted 10-fold with BPD and plated on CLA for direct enumeration. Plates were incubated at 42 °C under microaerophilic conditions for 48 h and enumerated for *Campylobacter* colonies as previously described by our laboratory.<sup>20</sup> All the experiments conducted in this study were approved by the Institutional Animal Care and Use Committee of the University of Arkansas.

### Chitosan Solution Preparation and Determination of Sub-Inhibitory Concentration (SIC)

The chitosan solution was prepared as mentioned pre-

viously.<sup>18</sup> The SIC of chitosan was determined using previously published protocol.<sup>21</sup> Briefly, 24 well polystyrene plates (Costar, Corning, NY, USA) containing CEB (2 mL/well) supplemented with two-fold dilutions of chitosan (0, 0.2, 0.1, 0.05, 0.025, 0.0125 and 0.00625%), were inoculated with ~5.0 log CFU of *C. jejuni* wild strain, followed by incubation at 42 °C for 24 h. Bacterial growth was determined by culturing on CLA agar plates. The highest concentration of chitosan that did not inhibit *C. jejuni* growth during mid-log (8 h), and stationary phase (24 h) were selected as the SIC for the compound.

#### RNA Isolation, cDNA Synthesis and Real-time Quantitative PCR

The effect of SIC of chitosan on the expression of *Campylobacter* genes critical for colonization in chicken was investigated using real-time quantitative PCR (RT-qPCR), as described previously.<sup>21</sup> The wild type *C. jejuni* strain was randomly selected from the four strains used in the *in vivo* trials for gene expression analysis. The strain was cultured with or without SIC of chitosan at 42 °C in CEB to mid-log phase (8 h) and total RNA was extracted using the RN easy Mini kit (Qiagen, Valencia, CA, USA), followed by complementary DNA synthesis (iScript cDNA synthesis kit, Bio-Rad). The cDNA synthesized was used as the template for RT-qPCR. The amplification product was detected using SYBR Green reagent (iQ SYBR Green Supermix, Bio-Rad). The primers for each gene (Table 1) were designed from published GeneBank *C. jejuni* sequences using Primer 3 software National Center for Biotechnology Information (NCBI) and synthesized from IDT DNA. The relative expression of candidate genes was determined using the comparative critical threshold ( $\Delta\Delta Ct$ ) method on a Quant Studio 3 real-time PCR system (Applied Biosystems, Thermo Fisher Scientific, Carlsbad, CA, USA). Data were normalized to the endogenous control (16S rRNA), and the level of expression of target genes between treated and untreated samples were analyzed to study effect of chitosan on expression of each gene. Duplicate samples

were used and the assay was repeated three times.

#### Statistical Analysis

Cecal *Campylobacter* counts were logarithmically transformed before analysis to achieve homogeneity of variance.<sup>22</sup> Analysis of the data was done using the PROC GLM procedure of SAS.<sup>23</sup> Treatment means were partitioned by LSMEANS analysis and probability of  $p < 0.05$  was required for statistical significance. Data comparisons for the gene expression study were performed using multiple t-test with GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California, USA, [www.graphpad.com](http://www.graphpad.com)).

## RESULTS

### Chitosan *in vitro*

*Campylobacter* counts were reduced by approximately 1 log at 2 and 4 h when co-incubated with 0.5% for all three molecular weights of chitosan as compared with controls (Table 2). At 8 h, all three chitosan preparations produced a 4.5 to 5 log reduction in counts when compared with controls.

### Chitosan *in vivo*

In trial 1, *Campylobacter* counts were reduced in six of the chitosan treatments: 0.25% and 0.5% Low Molecular Weight (LMW), 0.25% and 0.5% Medium Molecular Weight (MMW), 0.25% and 1% High Molecular Weight (HMW), in comparison to the positive control (Table 3). Trial 2 showed a significant reduction of *Campylobacter* by four of the chitosan treatments: 0.5% LMW, 1% LMW, 0.25% MMW, and 0.5% MMW (Table 3). Results from Trial 3 showed a significant reduction of *Campylobacter* by one of the chitosan treatments: 0.5% MMW (Table 3).

Gene with Accession no.	Primer	Sequence (5'- 3')	Gene description
<i>16S-rRNA</i> (NC_002163.1)	Forward Reverse	5'-TGAGGGAGAGGCAGATGGAA-3' 5'-TCGCCTTCGAATGGGTATT-3'	Ribosomal RNA (housekeeping gene)
<i>cadF</i> (NC_002163.1)	Forward Reverse	5'-CGCGGGTGTAATAATCCGTC-3' 5'-TCCTTTTGGCCACCAAAACCA-3'	Outer membrane fibronectin-binding protein
<i>jlpA</i> (NC_002163.1)	Forward Reverse	5'-AGCACACAGGGAATCGACAG-3' 5'-TAACGCTTCTGTGGCGTCTT-3'	Surface exposed lipoprotein
<i>ciaB</i> (NC_002163.1)	Forward Reverse	5'-TCTCAGCTCAAGTCGTTCCA-3' 5'-GCCCGCCTTAGAACTTACAA-3'	Invasion antigen protein
<i>fljA</i> (NC_002163.1)	Forward Reverse	5'-AGCTTTCACGCCGTTACGAT-3' 5'-TCTTGCAAAACCCAGAAGT-3'	Flagella biosynthesis RNA polymerase sigma factor
<i>motA</i> (NC_002163.1)	Forward Reverse	5'-AGCGGGTATTTTCAGGTGCTT-3' 5'-CCCCAAGGAGCAAAAAGTGC-3'	Flagellar motor protein
<i>motB</i> (NC_002163.1)	Forward Reverse	5'-AATGCCCAAGATGTCCAGCA-3' 5'-AGTCTGCATAAGGCACAGCC-3'	Flagellar motor protein

Table 1: Primers used for real time quantitative PCR (RT-qPCR) analysis.

Treatment	Time in hours			
	0	2	4	8
Positive controls	6.35*10 <sup>7</sup>	8.15*10 <sup>7</sup>	5.45*10 <sup>7</sup>	3.5*10 <sup>7</sup>
Low Molecular Weight	3.42*10 <sup>7</sup>	6.8*10 <sup>6</sup>	1.24*10 <sup>6</sup>	3.0*10 <sup>2</sup>
Medium Molecular Weight	8.55*10 <sup>7</sup>	2.55*10 <sup>6</sup>	1.82*10 <sup>6</sup>	5.5*10 <sup>2</sup>
High Molecular Weight	7.45*10 <sup>7</sup>	2.59*10 <sup>6</sup>	2.00*10 <sup>6</sup>	6.5*10 <sup>2</sup>

<sup>1</sup>0.5% concentration of: low molecular weight chitosan is 50-190 kDa; medium molecular weight chitosan is 190-310 kDa; or high molecular weight chitosan is 400-600 kDa, in 50 mM acetic acid.

<sup>2</sup>*Campylobacter* inoculum was added to each chitosan treatment and sampled at 0, 2, 4, and 8 h; samples were plated and enumerated after 48 h incubation.

<sup>3</sup>Values represent average campylobacteriosis counts of two separate replicate trials.

**Table 2:** The effect of different molecular weight chitosans on growth of *Campylobacter jejuni* *in vitro*<sup>1,2,3</sup> *Campylobacter* counts, *in vitro*.

	Chitosan dose	Trial 1	Trial 2	Trial 3
Positive controls Control	0%	8.77±.17 <sup>a</sup>	7.05±.69 <sup>a</sup>	8.36±.24 <sup>a</sup>
Low Molecular Weight	0.25%	7.06±.58 <sup>cde</sup>	7.1±.29 <sup>ab</sup>	8.59±.20 <sup>a</sup>
	0.5%	7.68±.27 <sup>bcd</sup>	3.96±1.02 <sup>c</sup>	7.88±.38 <sup>ab</sup>
	1.0%	7.96±.15 <sup>abc</sup>	ND <sup>d</sup>	7.76±.40 <sup>ab</sup>
Medium Molecular Weight	0.25%	6.76±.34 <sup>de</sup>	4.83±1.08 <sup>bc</sup>	8.47±.21 <sup>a</sup>
	0.5%	7.4±.38 <sup>bcd</sup>	3.25±.94 <sup>c</sup>	7.28±.70 <sup>b</sup>
	1.0%	8.03±.14 <sup>abc</sup>	7.45±.34 <sup>a</sup>	8.57±.17 <sup>a</sup>
High Molecular Weight	0.25%	7.45±.19 <sup>bcd</sup>	7.49±.31 <sup>a</sup>	8.16±.29 <sup>ab</sup>
	0.5%	8.43±.18 <sup>ab</sup>	7.8±.35 <sup>a</sup>	8.34±.26 <sup>a</sup>
	1.0%	6.3±.74 <sup>e</sup>	7.31±.30 <sup>a</sup>	8.51±.19 <sup>a</sup>

<sup>1</sup>Low molecular weight chitosan is 50-190 kDa; medium molecular weight chitosan is 190-310 kDa; high molecular weight chitosan is 400-600 kDa.

<sup>2</sup>ND: non-detectible.

<sup>3</sup>Day-of-hatch birds were fed chick starter treatments of 0.25%, 0.5% or 1% of either low molecular weight, medium molecular weight or high molecular weight chitosan, respectively, for the entire 15-day study; bird were inoculated with *Campylobacter jejuni* mixture on Day 6 and cecal contents were collected on Day 15 for campylobacteriosis enumeration.

<sup>4</sup>Means within columns with no common superscript differ significantly (p<0.05).

**Table 3:** The effect of different concentrations and molecular weight chitosans on cecal *Campylobacter jejuni* counts (means±SEM) in 15-day old broiler chicks during three separate trials<sup>1,2,3,4</sup> *Campylobacter* counts, *in vivo*.

## SIC and Gene Expression Analysis

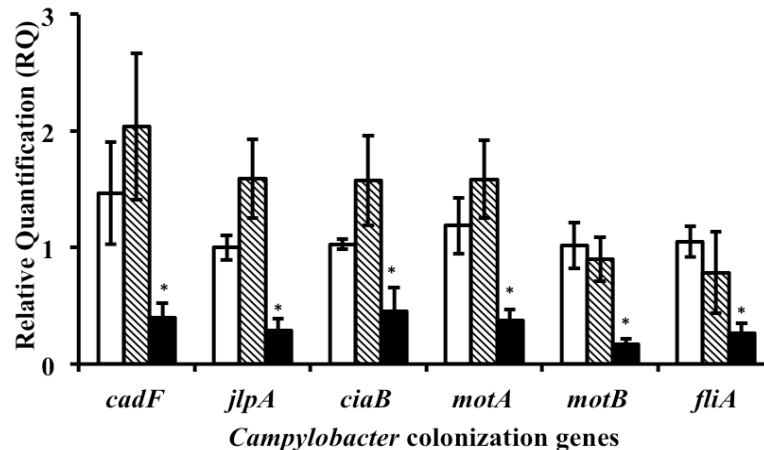
Since MMW chitosan was found to consistently reduce *Campylobacter* counts *in vivo*; we selected MMW chitosan for the gene expression analysis. One of the *C. jejuni* wild strain used in the *in vivo* study was randomly selected for the mechanistic study. Based on growth curve results (data not shown), the SIC of MMW chitosan that did not inhibit *C. jejuni* strain growth as compared to control was 0.0125%. This concentration of MMW chitosan was used for subsequent gene expression analysis. RT-qPCR results (Figure 1) revealed that MMW chitosan significantly reduced the transcription of genes coding for *Campylobacter* motility; namely, *fliA*, *motA*, *motB* and adherence (*cadF*, *jlpA*, *ciaB*) as compared to control (P<0.05). The expression of chicken colonization genes was not significantly

affected by acetic acid (P>0.05).

## DISCUSSION

Preliminary *in vitro* results utilizing a 0.5% dose demonstrate that the three molecular weight chitosan treatments reduce *Campylobacter* counts in comparison to the untreated controls (Table 2). To evaluate the ability of chitosan to reduce enteric *Campylobacter* colonization in chickens, the 0.5% concentration of all three molecular weight chitosans, plus a lower (0.25%) and higher dose (1%) were also evaluated. In the first trial, cecal *Campylobacter* counts were reduced in 6 out of 8 of the treatments (Table 3). When conducted in a second trial, 4 of the 8 treatments were effective; whereas in the third replicate trial, the 0.5% MMW reduced enteric *Campylobacter* counts





<sup>1</sup>RNA from wild type strain cultured either in the presence or absence (control) of 0.0125% chitosan to mid-log (8 hour) was used for cDNA synthesis and gene-expression analysis. 16S-rRNA was used as endogenous control. \*Means that differ significantly from the control (P<0.05).

**Figure 1:** Effect of 0.0125% MMW chitosan on the expression of chicken colonization genes (means  $\pm$ SEM) in *Campylobacter jejuni*.<sup>1</sup>

when compared with controls (Table 3). Although there is variability between replicate trials, the 0.5% MMW chitosan dose consistently reduced *Campylobacter* in all three trials.

To determine the potential mechanism of action of chitosan, we investigated the effect of SICs of MMW chitosan on the expression of critical chicken colonization genes of *Campylobacter*. SICs of antimicrobials, including antibiotics are known to alter pathophysiology of microbes by modulating gene transcription.<sup>21,24-26</sup> In *C. jejuni*, the flagellar biosynthesis gene, *fliA* regulates a large number of genes involved in motility, protein synthesis and colonization.<sup>27</sup> A mutation in *fliA* has been shown to reduce motility and colonization potential in chicken cecum.<sup>28</sup> Similarly, *motA*, *motB* are critical for flagella motor function and facilitate motility and colonization.<sup>27</sup> *CadF* is another important virulence gene that encodes a 37 kDA outer membrane protein, that along with *CiaB* and *JlpA*, promotes adherence to intestinal cells and colonization in the avian intestinal tract.<sup>27,29</sup> We observed that SIC of MMW chitosan significantly decreased the expression of motility genes as well as adherence genes as compared to control (Figure 1), indicating that the anti-colonization effect of chitosan could be potentially mediated through reduced transcription of critical genes.

The importance of replicating results demonstrating a significant reduction in enteric *Campylobacter* counts in pre-harvest poultry cannot be underestimated. Previous research conducted by our laboratory<sup>15,20,30</sup> and others<sup>31-33</sup> have highlighted the variability between trials when evaluating pre-harvest treatments against enteric *Campylobacter*. Because of this inherent variability associated with *Campylobacter* colonization studies, results from a single pre-harvest study may not fully evaluate the consistency or effectiveness of a *Campylobacter* intervention strategy.<sup>34-37</sup>

Feed application of chitosan is a viable application for reducing *Campylobacter* colonization in chickens; however, water application is also a possible option. Unfortunately, chitosan is insoluble in water within the normal pH range.<sup>38,39</sup> This problem can be resolved by mildly acidifying the water, as accomplished in our *in vitro* studies. It is possible this will enhance the efficacy of chitosan as proposed by Qin and co-workers.<sup>39</sup> Acidifying water lines is already being performed in some poultry operations, which can reduce another foodborne pathogen, *Salmonella*;<sup>22</sup> thereby, this aids in the reduction of *Campylobacter* as well in the water lines and during feed withdrawal prior to processing, without altering the gut epithelium.<sup>40,41</sup> Thus, acidifying water in poultry houses could have a number of positive effects on bird health and reduce the potential zoonotic transfer of pathogens to humans. This possibility is currently under investigation.

The use of pre-harvest intervention strategies to reduce *Campylobacter* colonization (e.g., chitosan) can be part of a multifaceted approach to reduce the incidence of this foodborne pathogen. It has been proposed that a 2-log reduction in *Campylobacter* on the chicken carcass could reduce the risk of human campylobacteriosis by up to 30-fold.<sup>42</sup> Perceptibly “small” reductions of *Campylobacter* in chickens could result in large reductions of campylobacteriosis incidences in humans. Olson and colleagues compiled data relevant to the consistent rise of campylobacteriosis incidences from the 1980’s through 2006 as occurred in many countries, including Denmark, England, Wales, Norway, Sweden, New Zealand, and Australia, many of which are currently monitored by the European Centre for Disease Prevention and Control (ECDC).<sup>43</sup> In the 2000’s, New Zealand focused on poultry as the primary source of *Campylobacter*; and by applying required regulatory implementations, along with the assistance of voluntary interventions, New Zealand saw a 54%

decline in campylobacteriosis incidences in 2008, compared to the period 2002-2006.<sup>44</sup> This decline was associated with a reduction in *Campylobacter* counts in chicken meat.<sup>45</sup> New Zealand's well-documented reduction of campylobacteriosis cases sets precedence for global reduction of *Campylobacter* by focusing intervention strategies on the poultry industry.

In conclusion, enteric *Campylobacter* counts were consistently reduced by in-feed supplementation of 0.5% MMW chitosan in three replicate trials. The use of this chitosan in pre-harvest poultry may be incorporated into a multifaceted strategy to reduce *Campylobacter* counts in chickens. Also, chitosan can be used to further reduce or inhibit *Campylobacter*s surviving on the chicken carcass or meat. Further studies are warranted to explore the potential use of chitosan for reducing *Campylobacter* contamination in pre- and post-harvest poultry and the potential mechanism of action through whole transcriptome analysis.

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#### CONFLICTS OF INTEREST

Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the USDA and does not imply its approval to the exclusion of other products that are suitable.

The authors declare that they have no conflicts of interest.

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