CPS CENTER for PRODUCE SAFETY

CPS 2017 RFP
FINAL PROJECT REPORT

Project Title
Application of chitosan microparticles to eliminate foodborne pathogens in agricultural water that contacts fresh produce

Project Period
January 1, 2018 – December 31, 2018 (extended to January 31, 2019)

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Objectives
1. *Investigate practical pre-harvest application of chitosan microparticles (CM) for pathogen reduction in the complex medium of irrigation pond water.*
2. *Optimize use of CM films for post-harvest application to reduce/prevent contamination of pathogens on produce.*
3. *Conduct preliminary experiments to investigate CM activity against norovirus.*
4. *Assess cost-effectiveness of chitosan application to agricultural water.*

Funding for this project provided by the Center for Produce Safety through:
Florida SCBGP grant# 024843

FINAL REPORT

Abstract
Water that is used for irrigating or washing produce is a food safety concern because it can become contaminated with foodborne pathogens. This study was conducted to determine the effectiveness of chitosan microparticles (CM) against the growth and survival of *Salmonella enterica* and *Escherichia coli* bacteria in natural water systems. CM is derived by sonicating chitosan (a solubilized form of chitin), and shows increased antimicrobial activity compared with unprocessed chitosan. CM activity was assessed for stationary phase *Salmonella* inoculated into various water sources, as well for natural populations of both *Salmonella* and *E. coli*. CM concentrations between 0.01 and 0.005% wt:vol consistently reduced inoculated *Salmonella* (3.7 log CFU/mL) to non-detectable levels within 24 h post-treatment for autoclaved pond water or in isotonic glucose. These effects were consistent for different chitosan formulations, and all CM preparations were relatively stable at 4°C for over 2 months, but activity was lost upon frozen storage. In addition, CM treatment effectively eliminated inoculated and natural populations of *Salmonella* for intact pond water that was obtained from multiple sources at different time points. Fecal indicator bacteria (i.e., generic *E. coli*) were also reduced to non-detectable levels following CM treatment. One exception was for water from an urban creek, which showed reduced anti-*Salmonella* efficacy at one time point; however, this water source also had the highest level of fecal indicator bacteria. Furthermore, signicant reducations in viral RNA were demonstrated for both murine norovirus and a surrogate MS2 bacteriophage afterfollowing CM treatment compared to controls. Thus, the research herein presents the first comprehensive examination of CM activity in the complex medium of natural water collected from pond, creek or lake systems. These data demonstrated that CM presents a relatively safe and biodegradable alternative for antimicrobial treatment of natural water systems contaminated with potential human pathogens.

Background
Water that contacts produce products before harvest or during processing has been implicated as a source of pathogen contamination and a food safety concern (CDC, 2012; Pachepsky et al., 2011). Environmental surveys have found pathogens are frequently associated with aquatic sources and may persist over long periods of time (Gu et al., 2013a, 2013b; Li et al., 2014; McEgen et al. 2014; Uesugi et al., 2007), and several outbreaks have been attributed to contaminated wash water. Furthermore, current metrics that reflect fecal contamination may not accurately ensure the absence of pathogens (Luo et al., 2015; McEgan et al., 2014), particularly those that may be derived from non-fecal sources (i.e., soil, water, foliage). Therefore, the agriculture industry is increasingly relying on disinfectants for water treatment and prevention of pathogen contamination. Typical treatments include sodium hypochlorite, chlorine dioxide, and peroxyacetic acid (Park and Kang, 2015; Suslow et al., 2003; Uyttendaele et al., 2015; Yuk, H.-G. et al., 2006; Zhang et al., 2015). Unfortunately, the efficacy of these treatments can be limited by concentrations of organic matter that overwhelm their antimicrobial capacity. The potential toxicity of these compounds can also necessitate additional costs related to their disposal (FDA, 2014). Thus, novel approaches are needed to ensure the safety of agricultural water and provide mitigation for water resources that do not meet current FDA standards.

A possible candidate for sanitation of agricultural water is the soluble derivative of the second-most abundant biopolymer on earth, namely chitin. Chitin is the primary component of the exoskeletons of crustaceans, algae, and insects, and is also a readily available and relatively inexpensive byproduct of the seafood industry. Chitosan [poly-(b-1/4)-2-amino-2-deoxy-D-glucopyranose] is a more soluble form of chitin, composed of collective derivative compounds

with variable size and deacetylation. It has demonstrated a broad range of antimicrobial activity in numerous studies; furthermore, chitosan is "generally ecognized as safe," or GRAS, by FDA for certain food applications (Harish Prashanth and Tharanathan, 2007; Kurita, 2006). It is also commonly used for clarification and removal of heavy metals in wastewater directed to settling/holding ponds where chitosan (Liqui-Floc) causes fine sediment particles to bind together with subsequent removal by sand filtration. Application of chitosan to vegetable washing process was found to remove up to 100% of particulates when used in conjunction with sand filtration at pH 5 (Van Haute et al., 2015).

Antimicrobial properties of chitosan can vary with molecular weight (Jia et al., 2001; Raafat and Sahl, 2009b; Raafat et al., 2008) and the method of formulation (Kong et al., 2010). In general, lower molecular weight and higher degree of deactylation improve antimicrobial activity. The difficulty with using chitosan as a treatment for irrigation water stems from its inactivity in the neutral pH range (Chung et al., 2003; Li et al., 2002). This limitation is overcome by processing chitosan into microparticles (CM) which retain activity at neutral and above pH (Jeon et al., 2014; Jeong et al., 2011). CM was shown to have effective antimicrobial activity against Shiga toxin–producing *Escherichia coli* (STEC), *Salmonella,* and *Vibrio cholerae* (Jeon et al., 2014). Indeed, therapeutic application of CM was evidenced by reduced shedding of STEC in cattle after treatment with CM (Jeong et al., 2011). Unlike many antimicrobials, bacteria treated with CM did not develop resistance to CM or other antibiotics (Ma et al., 2016). CM was also an effective treatment in seawater and live oyster processing for elimination of multiple *Vibrio* spp. (Fang et al., 2015); antibacterial activity of CM reduced bacterial levels in seawater by >7 log CFU/ml, and significant reductions ($p < 0.001$) were observed in oysters treated with CM compared to untreated controls. Recent studies demonstrated significant reductions for various serotypes of *Salmonella*, with minimum inhibitory concentrations ranging from 0.001 to 0.025% (wt:vol) CM (Fan et al., 2017).

The research herein examined the application of CM as a possible treatment for irrigation water and/or agricultural wash water. Unfortunately, prior research indicated that CM activity was reduced for stationary compared to log phase cells. Also, application of CM to more complex mixed communities, such as pond water, required higher concentrations that may not be practical or cost effective for agricultural applications (Fan et al., 2017). Therefore, the present research assessed the antimicrobial activity of CM for stationary phase *Salmonella* inoculated into pond water from various sources. Commercial application of chitosan to irrigation and produce sanitation presents an opportunity to apply a biodegradable and non-toxic seafood refuse resource as an economical food safety solution, and this research aimed to inform the agricultural community about specific parameters and limitations for this application.

Research Methods

Preparation of chitosan microparticles (CM). In the present research, three formulations of chitosan were used for the preparation of CM: CMA and CMC were prepared from chitosan purchased from Primex (#TM5017 and TM4815, respectively), and CMB (#0005RM) was from Pharmanutrient (**Table 1**). These chitosan formulations differed somewhat in various parameters but with the same degree of deacetylation. Chitosan microparticles were prepared as described previously (Jeon et al., 2014). Briefly, a chitosan solution (2% w/v) was prepared in 2 L of 2% acetic acid (v/v) and 1% tween 80 (v/v). For cross-linking, the chitosan solution was stirred and a 10% solution of sodium sulfate (w/v) was added dropwise during 25 min of sonication at ~40 watts; the sonication process was continued for an additional 25 min. The CM preparation was collected by centrifugation (8200 x g for 10 min) and washed with sterile water 3 times, resuspended in 200 ml of sterile water, and assessed for sterility. After preparation, the

mass of the CM was measured by weighing a 1-mL solution that had been dried overnight in an oven, for a yield of ca. 0.05 g/mL. The prepared solution was stored in a 50-mL conical tube at 4°C to yield a concentration of stock solution at ca. 5% (wt:vol).

Inoculum preparation. Unless otherwise noted, the strain of *Salmonella enterica* used in this study was wild-type serovar Typhimurium ATCCBAA14028S (14028). Strain comparisons were performed with an avirulent surrogate strain MHM112 of *S.* Typhimurium, lacking all pathogenicity islands and the *Salmonella* virulence plasmid (de Moraes et al., 2016), as well as with other serotypes. Bacteria were retrieved from frozen stock (−80°C) and grown overnight in Luria broth with NaCl (LBN) prepared with 1% tryptone,1% yeast extract, and 1% NaCl at 37°C and 200 rpm. Overnight cultures were washed 3 times and diluted in de-ionized water to prevent the inhibition of chitosan microparticle activity by NaCl as previously reported (Fan et al., 2017).

Minimum inhibitory concentration (MIC). Sensitivity *of Salmonella* to CM was determined by MIC using macrodilution broth method, as previously described by Clinical and Laboratory Standards Institute (CLSI 2012) with slight modifications. Briefly, MIC is defined as the lowest concentration of CM that inhibits visible growth of *Salmonella* after overnight incubation. The protocol in this study used culture in 5 ml of lactose broth (LacB) instead of Mueller-Hinton broth. Bacterial inocula were standardized to final concentration of ca. 10^3 CFU/mL. Serial dilutions (1:2) were made of CM (0.1 to 0 0.000078% [w/v] in LacB [pH 7]). Tubes were incubated at 37°C overnight with shaking (200 rpm).

Survival of *Salmonella* in CM-treated water and media. Solutions of CM in sterile de-ionized water, isotonic solution (0.264 M glucose), and autoclaved or untreated natural surface water (50 mL each) were prepared in a 250-mL flask. Surface water samples were collected at various sites in Gainesville, FL; these sites included Lake Alice, Paynes Prairie, Lake Wauburg, Bivens Arm, Marston Pond, and Tumblin Creek. Samples were collected before midday and immediately transported to the lab. Water was used within 24 h or stored for later use at 4°C. De-ionized and isotonic water were adjusted to pH 7; pond water pH ranged from 6.5 to 8. CM stock was added into each flask to achieve the desired final concentration. Following the addition of CM, each flask was inoculated with stationary phase culture for a final concentration of ca. 10^3 CFU/mL, as described above. Flasks were incubated at room temperature without shaking overnight. Cultures were plated onto XLT4 and incubated at 37°C at the time points of interest to determine CFU/mL.

Survival of *Salmonella* on tomato stem scars following treatment with CM. *Salmonella* inocula were prepared as described above or using rifampicin-resistant strain grown with in Luria broth (LB) with rifampicin (80 ppm). Inoculum (100 µl) was applied to the tomato stem scar (n=6) and dried for 2 h in a fume hood at room temperature. Stem scars were excised aseptically and resuspended in 5 mL of de-ionized (DI) water or PBS with or without 0.1% CM and incubated for 24 h at room temperature. Each sample (in a Whirl-Pak bag) was smashed with a mallet on the lab bench and then processed in a stomacher for 2 min. Surviving bacteria in the filtrate were enumerated on either XLT4 or TSA with 80 ppm rifampicin after overnight incubation at 37°C.

EC-MUG assay for the detection of generic *E. coli*. Pond water was collected from four locations: Marston Pond, Bivens Arm, Tumblin Creek, and Paynes Prairie. Flasks were prepared in triplicate with 50 mL of pond water and 0.01% CM. Cultures were then incubated at benchtop for 24 h. Following incubation, a set of 9 culture tubes with 10 mL of Laurel tryptose broth were prepared for each pond water sample for the presumptive phase of the EC-MUG assay. Three tubes in each set were inoculated with 1 mL, 0.1 mL, and 0.01 mL of pond water. Final MPN was multiplied by 10 to account for inoculation size. All tubes were incubated at 35°C (± 0.5°C) for 48 h. After this incubation period, all tubes were evaluated for the presence of

coliforms by checking for turbidity and gas formation in the Durham tubes. For the confirmatory phase, tubes of 10 mL each of EC-MUG broth were prepared. Each tube with positive results from the presumptive phase were used to inoculate tubes of EC-MUG broth with an inoculation loop. All tubes in this phase were incubated in a water bath at 45°C (± 0.5°C) for 48 h. After the incubation period, all tubes were evaluated for *E. coli* by checking for turbidity, gas formation, and fluorescence under a UV lamp. To expand the limit of detection, cultures that were negative on XLT4 were also inoculated into 10 mL of 2x buffered peptone water and incubated at 37°C for 18 h. Following incubation, positive tubes were incubated in RV10 broth at 42°C for 48 h.

Antiviral activity of CM. To assess the antiviral efficacy of CM, male-specific bacteriophage (MS2) was used as it is also an RNA non-human pathogen virus with a size and shape similar to that of the norovirus. CM was produced by ion gelation, involving cross-linking with sodium sulfate, sonication, and washing with de-ionized water. A 10 $\log_{10}$ plaque forming units (PFU)/mL stock of MS2 was added to the 0.3% CM in PBS (w/v) for a final titer of 7 $\log_{10}$ PFU/ml, and incubated for 0, 1, 2, 3 and 20 h at room temperature while gently shaking. The infectious titer of MS2 was quantified by plaque assay using the host *E. coli*. Similarly, RT-qPCR was performed on RNA extracted from the MS2 following the treatment with 0.3% CM. A calibration curve established from the serially diluted MS2 genome obtained was then utilized to determine the titer of the viral genome in the treated samples. Antiviral activity of CM (0, 0.01%, 0.1% and 0.3%) was also assessed using murine norovirus (MNV) at high (10^7 $TCID_{50}$) and moderate (10^4 $TCID_{50}$) titers. Virus concentration was quantified using RT-qPCR.

Viral binding to CM. In addition, the ability of MNV to bind to chitosan microparticles was also assessed. Varying concentrations of CM (0, 0.01%, 0.1% and 0.3%) were incubated with 10^7 $TCID_{50}$ of MNV for 3 h at room temperature. After incubation, the mixture was centrifuged to pellet CM and the supernatant was transferred to a clean tube. RNA lysis buffer was added to both the CM pellet and supernatant and MNV was quantified using RT-qPCR.

Statistical analysis. Data were analyzed using SAS JMP pro 14. Student's t-test was performed to test the null hypothesis that there was no difference between treatments. Analysis of variance (ANOVA) was used to compare means of log reduction over time aged in pond water. Analysis of means (ANOM) was used to compare differences among *Salmonella* strains in pond water. Experiments were conducted in triplicate unless otherwise specified. Significance was determined at $p < 0.05$.

Research Results

Effects of chitosan formulation, solvent, and storage on CM activity. Minimum inhibitory concentration (MIC) of CM for *Salmonella* was used to compare activity of CM prepared from different sources of chitosan. The MIC for CM preparations varied somewhat based on the source of chitosan, as shown in **Table 2**: CMB (Pharmanutrients) and CMC (Primex) showed somewhat lower MIC compared to CMA (Primex), while CMC was stable in refrigerated storage for a longer period compared to the CMA or CMB. Freezing at −20°C resulted in significant loss of activity after 2 weeks of storage (data not shown). Survival of stationary phase *Salmonella* in monoculture after 24-h exposure to CM prepared in either autoclaved pond water, de-ionized water or isotonic solution was compared. Results for an isotonic solution of CM and CM in autoclaved pond water were similar in that bacterial levels were reduced to near or below the limit of detection (LOD) for both 0.01% and 0.05% CM (**Figure 1**). Conversely, levels of *Salmonella* were not significantly reduced at these concentrations in de-ionized water. Indeed, concentrations as high as 0.3% CM were unable to significantly reduce levels of *Salmonella* in this medium (data not shown).

Effects of CM on survival of stationary phase *Salmonella* in natural water communities. To assess the practical application of CM as a mitigation strategy against *Salmonella* in irrigation water and/or agricultural waste water, studies of anti-*Salmonella* activity of CM focused on intact pond water. Prior studies indicated that CM activity was reduced for stationary compared to log phase cells (Fan et al., 2017). Since stationary phase may be the primary state for bacteria in the environment, these studies also focused on stationary phase cells. As shown in **Figure 2**, activity of CM against survival of stationary *Salmonella* varied somewhat with the particular chitosan formulation used to generate CM. Overall, CMC preparation performed better than CMA or CMB derived from the other formulations of chitosan. Application of CMC resulted in reduction of *Salmonella* levels to below the LOD at concentrations as low as 0.005% (w/v), whereas CMB required 0.01% and CMA approached but did not reach the LOD. At lower concentration of CM (0.0025%), reductions in *Salmonella* levels were less pronounced but still significant for CMA and CMC but not for CMB. Treatments that resulted in reduction below the LOD were assessed for re-growth after 1-week incubation, and none was detected. Attempts to scale up CM treatment using larger volumes (50, 100, 150, and 1000 mL) showed similar reductions in levels of *Salmonella* following treatment, and all samples were below the LOD with 0.005% CM after 24-h exposure (data not shown). Prior studies (Fan et al., 2017) used fairly high inocula (10^5–10^7 CFU/mL) to examine anti-*Salmonella* activity of CM; however, these levels are not commonly found in agricultural environments (Luo et al., 2015). Therefore, inoculum size of *Salmonella* was examined as a variable using 0.005% CM. Results demonstrated the CM effectively reduced stationary phase *Salmonella* to non-detectable levels for inocula ranging from 10^2 to 10^5 CFU/ml (**Figure 3**). These data indicate that CM is capable of producing a ~5-log CFU/mL reduction of *Salmonella* in pond water upon treatment.

Effects of surface water source and serotype on anti-*Salmonella* activity of CM. Under field conditions, physical and biological conditions in irrigation pond may vary widely. Therefore, the efficacy of CM activity was examined for different sources of water samples, including three ponds, a lake, and an urban creek. Identical results were obtained for all samples tested (**Figure 4**), and in each case, *Salmonella* levels were reduced to below the LOD at the lowest concentration tested (0.01% CM). Different serotypes were previously shown to vary somewhat in their sensitivity to CM, as determined by MIC (Fan et al., 2017). Therefore, serotype variation was examined for any possible effect on survival of *Salmonella* in pond water. Response to CM varied somewhat by serotype (**Figure 5**), and while there were significant differences between some serotypes, no one serotype differed significantly from the mean of all serotypes. Interestingly, this study demonstrated significant differences in survival of *Salmonella* depending on the water source, and in this instance, CM activity was greatly decreased in water samples from Tumblin Creek, as compared to the other water sources.

Stability of the anti-*Salmonella* activity of CM in pond water. The residual stability of CM treatment for pathogen reduction in pond water is an important measure of its effectiveness over time, especially for irrigation pond water applications where continued exposure to environmental sources of *Salmonella* could occur frequently. To determine the degree to which CM activity was reduced over time, *Salmonella* was inoculated into pond water at 24 and 48 h post addition of CM (0.01%) to the water. After 1-day exposure to pond water, CM retained its ability to reduce *Salmonella* to non-detectable levels (**Figure 6**). At 2 days exposure, however, levels of *Salmonella* in water treated with CM were not significantly different from those in untreated water.

Treatment of surface water with CM results in a significant reduction of coliforms. Total and fecal coliforms have been used as indicator organisms for the detection of human waste contamination for decades, the latter having a strong correlation with the development of

gastrointestinal illnesses (Dufour, 1984). As such, it is important for a proposed treatment to affect indicator organisms as well as the pathogens of interest. In **Table 3**, we see the results of an EC-MUG assay performed in different bodies of water before and after treatment with 0.01% CM. Invariably, there was significant reduction ($p < 0.05$) of generic *E. coli*.

Treatment of tomato stem scars with CM for reduction of *Salmonella.* Initial experiments did not show reduction of *Salmonella* after CM (0.1%) treatment; however, we found high levels of background contamination in the uninoculated samples. Experiments are in progress with a rifampicin-resistant strain to reduce background levels on plates.

Antiviral efficacy of chitosan microparticles. In the presence of 0.3% CM, the infectious titer of MS2 was immediately decreased by 3.5±0.6 $\log_{10}$ PFU up to 3 h. After 20 h, the reduction reached 4.5±0.0 $\log_{10}$ PFU. RT-qPCR data indicated an immediate reduction in viral genome of 4.0±1.1 $\log_{10}$ RT-qPCR units up to 3 h and appears to remain constant after 20 h (**Figure 7**). These findings reveal CM as a potential antiviral treatment against human norovirus. Further investigation into the mechanism of action and effects on contact surfaces may add more insight into the use of CM against human norovirus in water and on environmental surfaces. Similar results were observed with both high and moderate titers of the MNV surrogate in the presence of CM (**Figure 8**).

Viral binding to CM. Quantification of MNV from CM pellets revealed that the virus did not stringently bind to CM within 3 h, regardless of the concentration of CM used (**Figure 9**). All detectable virus remained in the supernatant of the MNV-CM mixtures.

Cost analysis of chitosan application. The material cost for CM was calculated as follows: Starting with 2 L of 2% chitosan (40 g), the solution was processed to prepare CM, and the final yield of the dry matter was 0.05 g/mL in a volume of 200 mL, for a total yield of 10 g or a 25.0% recovery of CM from chitosan. Therefore, 1 kg (cost = $47.75 from Primex) of chitosan will yield 250 g of pure CM after processing. To prepare an effective solution of 0.005% CM (0.05 g/L) would require 0.2 g/L of chitosan and cost $0.00955/L or $0.036/gallon. Cost of chitosan is above the range of costs of chlorination $0.00015–$0.005/gallon (personal communication with Dr. Paul Fisher, University of Florida) but could be adjusted with improved yield or efficacy.

Outcomes and Accomplishments

This research examined various parameters that could be relevant to the practical application of CM as a sanitizer for agricultural water systems. Chitosan solutions are broadly described as the water-soluble fraction of chitin, but these heterogeneous mixtures can vary greatly in their degree of acetylation, solubility, molecular weight, etc., depending on the method of preparation. Furthermore, these factors have been shown to influence antimicrobial activity, although reports on the effects of specific parameters are not always consistent (for a review see Kong et al., 2010). Therefore, the possible contribution of chitosan formulation on the antimicrobial activity of CM was tested for three chitosan products. All CM preparations were relatively stable at 4°C for >2 months, but activity was lost upon frozen storage, as determined by MIC. Overall, comparable results were obtained for all product samples, in that MICs did not vary more than 2-fold. However, CMC prepared from Primex chitosan gave more reproducible results for reduction of *Salmonella* in pond water, consistently bringing levels to below the LOD at concentrations of 0.01 and 0.005%. The chitosan products examined showed identical deacetylation and similar size parameters. Results obtained herein are also similar to prior research (Fan et al., 2017; Jeon et al., 2014; Jeong et al., 2016; Ma et al., 2016), which used Sigma chitosan (Sigma-Aldrich 448869−250G) with a lower degree of deacetylation (75−85%)

than preparations from this study. For example, the MIC for Typhimurium 14028 was 0.025% CM using Sigma chitosan (Fan et al., 2017), and in the present study the MIC ranged from 0.003 to 0.0125%. Thus, subtle changes in the size and deacetylation of chitosan, as well as long-term refrigeration, did not dramatically influence the activity CM against *Salmonella.*

Several studies have indicated that the solvent used to prepare CM can influence antimicrobial properties (Kong et al., 2010). Our previous work demonstrated that pH, salinity, and temperature altered activity, and that stationary cells were more resistant to CM as compared to log phase cells(Fan et al., 2017; Fang et al., 2015). The present study showed that bactericidal activity of CM for stationary phase *Salmonella* required an isotonic glucose solution, as CM was not effective in DI water alone. Maillard reactions of chitosan (heating with reducing sugars) enhances the degree and stability of antimicrobial activity by increasing the positive charge density (Kong et al., 2010). Other studies have shown greater activity of CM in DI water for log phase cells as compared to stationary phase bacteria. For example, minimal activity (2 log CFU/mL reduction) for stationary phase cells in DI water was reported even at concentrations of 0.1–0.3% CM (Fan et al., 2017). Furthermore, longer incubation time (48 h) was required to reach 99.9% reductions for log phase cells treated with CM prepared in DI. Tsai and Su (1999) suggested that loss of cell surface negativity in stationary phase may contribute to decreased susceptibility. Thus, a combination of increased positive charge for CM and decreased negative charge on the bacteria may function to negate CM activity for stationary phase cells in DI water.

CM activity in autoclaved pond water was as effective as CM in an isotonic solution, suggesting organic and/or inorganic matter contributed to CM efficacy. CM demonstrated consistent robust antimicrobial activity over different water sources, extended time periods, multiple *Salmonella* serotypes, and in larger volumes of water (up to 1 L). One exception was an observation for the Tumblin Creek sample, which showed reduced CM activity in one sample; however, this was the only flowing water system source with obvious changes in water clarity and color. Thus, anthropomorphic input, as well as environmental factors, may have contributed to the difference in CM activity for that particular sample.

The research herein presented the first comprehensive examination of CM activity in a complex medium of natural water collected from pond, creek, or lake systems. CM was consistently able to reduce *Salmonella* to non-detectable levels in various pond/lake/creek water samples. Likewise, CM was able to cause 3-fold reductions in levels of naturally occurring generic *E. coli* and coliform populations as determined by MPN. Current standards for irrigation water require generic *E. coli* at levels ≤126 most probable number (MPN)/100 mL (rolling geometric mean n=5), and ≤235 MPN/100 mL for any single sample will trigger additional testing. Two of our samples were ≤230 MPN/100 mL, and CM treatment reduced levels to 40 MPN/100 mL.

Summary of Findings and Recommendations

This project demonstrated that chitosan microparticles (CM) present a viable alternative for treatment of natural water systems contaminated with potential human pathogens of both bacterial and viral origin. The current method of preparation involves multiple centrifugations that may need to be scaled up or replaced with an alternative rinsing method in order to be practical for industrial applications. Advantages inherent to CM may justify possible additional cost: CM is a biodegradable, non-toxic alternative to reliance on harsh chemicals, such as chlorine, which may present issues with toxicity and disposal; additionally, the mode of action does not appear to promote antimicrobial resistance (Ma et al., 2016). Furthermore, the coagulant and flocculant properties of chitosan may contribute to removal of other unwanted components of water systems, such as heavy metals (Abebe et al., 2016; Bina et al., 2014; Raafat and Sahl, 2009a). Thus, CM has the benefit of dual applications: a flocculant and a broad-spectrum antimicrobial.

APPENDICES

Publications and Presentations

Publication (in preparation):
George, Andree, Amanda Mascarenhas, Andrea Sakelson, K.C. Jeong, and Anita C. Wright. "Chitosan microparticles effectively reduce *Salmonella enterica* and generic *Escherichia coli* in natural water samples." *In preparation – final draft is 95% complete (as of March 2019)*

Presentations:
George, A., A. Mascarenhas, K.C. Jeong, and A.C. Wright. 2019. "Chitosan microparticles effectively reduce *Salmonella enterica,* coliforms, and generic *Escherichia coli* in natural water samples." [Poster 06] Presented at the Emerging Pathogens Institute Research Day, University of Florida, Gainesville, FL; 12 February 2019.

Barnes, C., R. Barber, A. Wright, K.C. Jeong, and N. Montazeri. 2019. "Antiviral efficacy of chitosan microparticles on MS2 bacteriophage, a human norovirus surrogate." [Poster 02] Presented at the Emerging Pathogens Institute Research Day, University of Florida, Gainesville, FL; 12 February 2019.

Budget Summary

Total funds awarded to the project were $53,210. A budget summary as of the report submission date (March 1, 2019), is provided below:

Budget Category	Budget	Spent	Balance
Fringe Benefits	$3,180.00	$2,711.17	$468.83
OPS & Other Personnel	$32,100.00	$12,440.00	$19,660.00
Postdoctoral	$0.00	$17,644.30	-$17,644.30
Undergraduates	$0.00	$3,101.25	-$3,101.25
Other Expenses	$450.00	-$149.03	$599.03
Materials & Supplies	$9,760.00	$9,642.08	$117.92
Domestic Travel	$1,765.13	$1,738.03	$27.10
F&A	$2,822.00	$2,871.75	-$49.75

Tables and Figures

(see below)

Tables 1–3 and Figures 1–9

Table 1. Description of different formulations of chitosan used to prepare CM.

Parameters[a]	Chitosan Source[b]		
	CMA	CMB	CMC
Dry content	92.60%	N/A	91.40%
Turbidity	24 NTU	N/A	21 NTU
Viscosity	61 cP	≤ 100 cP	8 cP
Solubility	99.90%	≥ 98%	99.90%
Deacetylation	90%	90%	90%
Particle size	95% pass 18 mesh	95% pass 40 mesh	100% pass 100 mesh

[a] Parameters were assessed by the individual company. They are measured as follows:

i. Dry content: Ratio of dried chitosan mass to mass of chitosan before drying
ii. Turbidity: Cloudiness of the substance measured in nephelometric turbidity units (NTU)
iii. Viscosity: Resistance to flow measured – measured as centipoise(cP) equivalent to one milliPascal-second (mPa.s)
iv. Solubility: Dissolution of substrate into 1% acetic acid
v. Deacetylation: Degree of deacetylation of chitin upon treatment with NaOH – determined by colloidal titration
vi. Particle size: Percentage of particles passing through a wire mesh – mesh units are openings per inch, thus particle size is inversely proportional to mesh units

[b] Chitosan reagents TM5017 (CMA) and TM4815 (CMC) were obtained from Primex (Primex ehf), and 0005RM (CMB) was obtained from Pharmanutrients.

Table 2. Stability of different CM formulations with storage

Storage time (weeks)[a]	MIC (% w/v)[b]					
	CMA 4°C	Standard deviation	CMB 4°C	Standard deviation	CMC 4°C	Standard deviation
0	0.00625	N/A	0.003125	N/A	0.003125	N/A
2	0.00625	N/A	0.00625	N/A	0.003125	N/A
4	0.00625	N/A	0.00625	N/A	0.003125	N/A
6	0.00625	N/A	0.00625	N/A	0.00625	N/A
8	0.0125	N/A	0.00625	N/A	0.00625	N/A
10	0.00625	N/A	0.00625	N/A	0.010375	± 0.003
14	0.0125	± 0	0.00625	± 0		

[a] CM was stored at 4°C; storage time is measured as weeks of storage post preparation.
[b] MIC was determined as described in methods using *S. enterica* Typhimurium 14028.

Table 3. Effects of CM treatment on generic *E. coli* content in pond water

Pond	**Untreated**			**CM-Treated (0.01%)**		
	MPN (per 100 mL)[a]	Lower limit	Upper limit	MPN (per 100 mL)	Lower limit	Upper limit
Marston	230	40	1200	0	0	0
Bivens Arm	40	<5	200	0	0	0
Paynes Prairie	0	0	0	0	0	0
Tumblin Creek	2400	360	13000	40	<5	200

[a] MPN for generic *E. coli* was determined using EC-MUG assay, as described in methods.

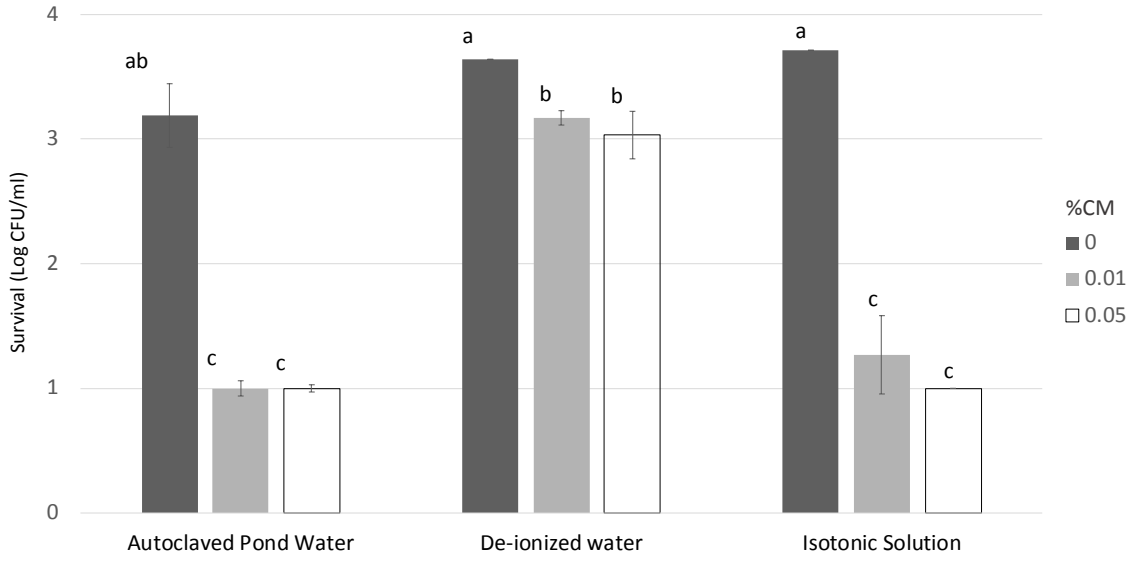

Figure 1. Effects of solvent on anti-*Salmonella* activity of CM. *S. enterica* Typhimurium 14028S was inoculated (ca. 3.7 log CFU/mL) into CM solutions (CMC formulation) prepared in 250-mL flasks containing 50 mL of sterile pond water from all ponds, deionized water, or isotonic glucose (0.264 M). Survival of *Salmonella* was assessed for CMC concentrations of 0, 0.01, or 0.05% (wt:vol) after 24 h by plate count on XLT4 agar. Error bars indicate standard error of the mean for three experiments. Limit of detection (LOD) was 1.0 log CFU/mL. Different lowercase letters represent significant differences at $p < 0.05$ among solvents at the same concentration of CM.

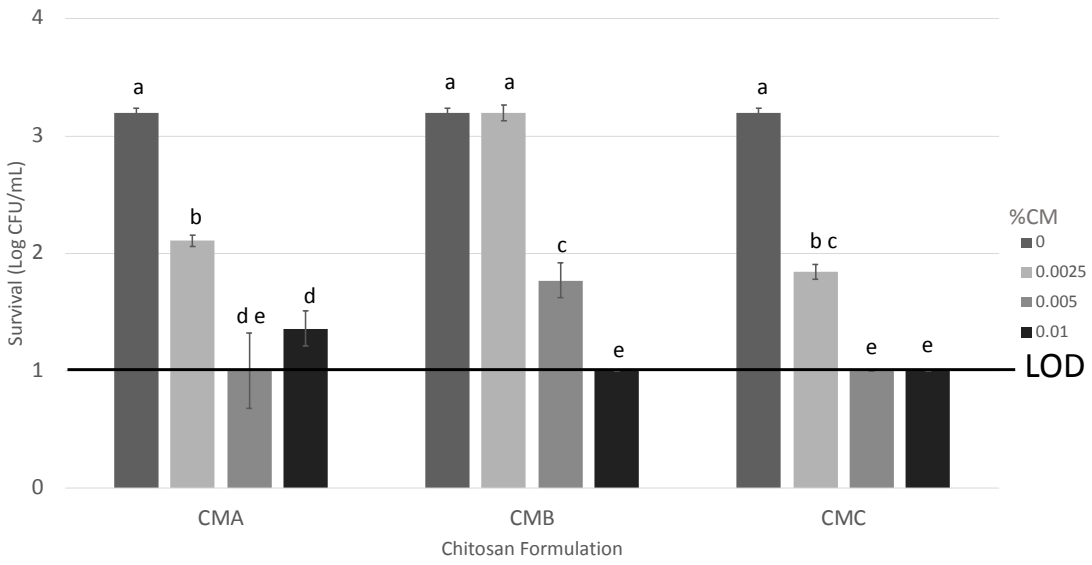

Figure 2. Effects of chitosan formulation on anti-*Salmonella* activity of CM in pond water. *S. enterica* Typhimurium 14028S was inoculated (ca. 3.7 log CFU/mL) into CM solution prepared with water collected from Paynes Prairie. Different formulations of CM (CMA,CMB, and CMC) were prepared from different chitosan products at concentrations of 0, 0.0025, 0.005, or 0.01% (wt:vol). Survival of *Salmonella* after 24h was assessed by plate count on XLT4 agar. Error bars indicate standard error of the mean for three experiments. Limit of detection (LOD) was 1.0 log CFU/mL. Different letters represent significant differences at $p < 0.05$.

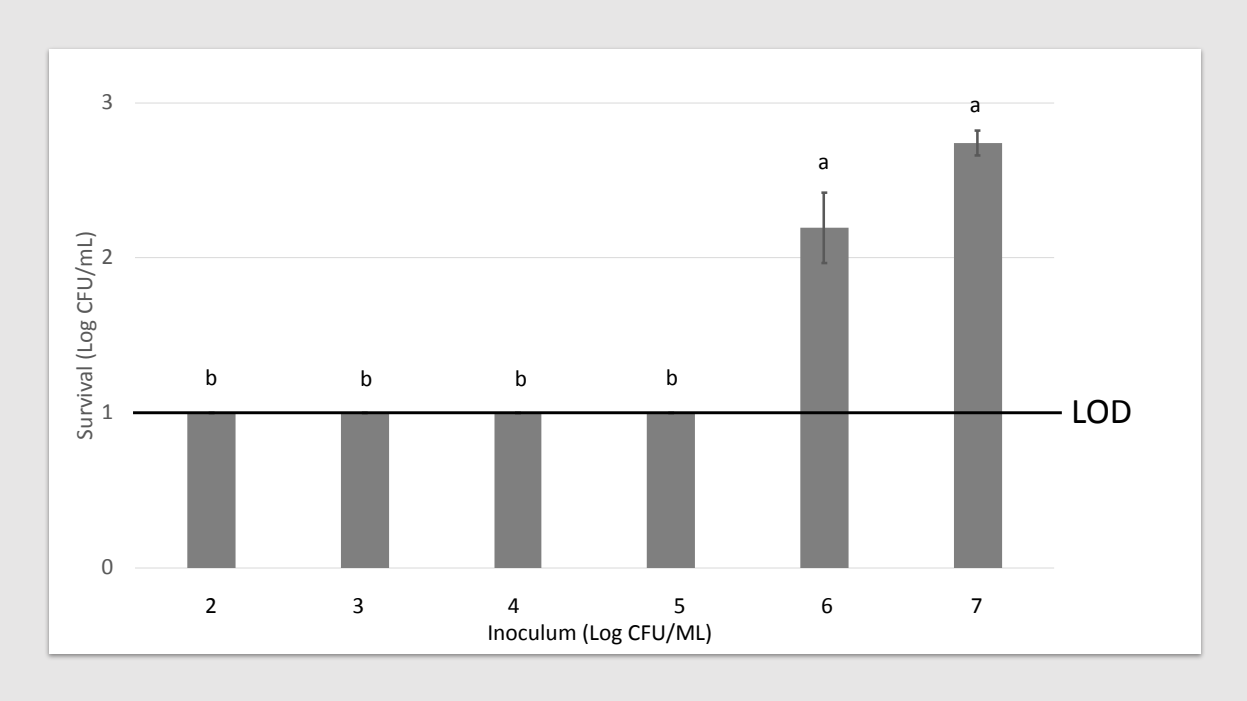

Figure 3. Effects of inoculum size on anti-*Salmonella* activity of CM in pond water. *S. enterica* Typhimurium 14028S inocula ranged from ca. 2 to 7 log CFU/mL. Bacteria were inoculated in 250-mL flasks containing 50 mL of 0.005% CMC in pond water (Paynes Prairie). Survival after 24 h was assessed by plate count on XLT4 agar. Error bars indicate standard error of the mean for three experiments. Limit of detection (LOD) was 1.0 log CFU/mL. Different letters represent significant differences at $p < 0.05$.

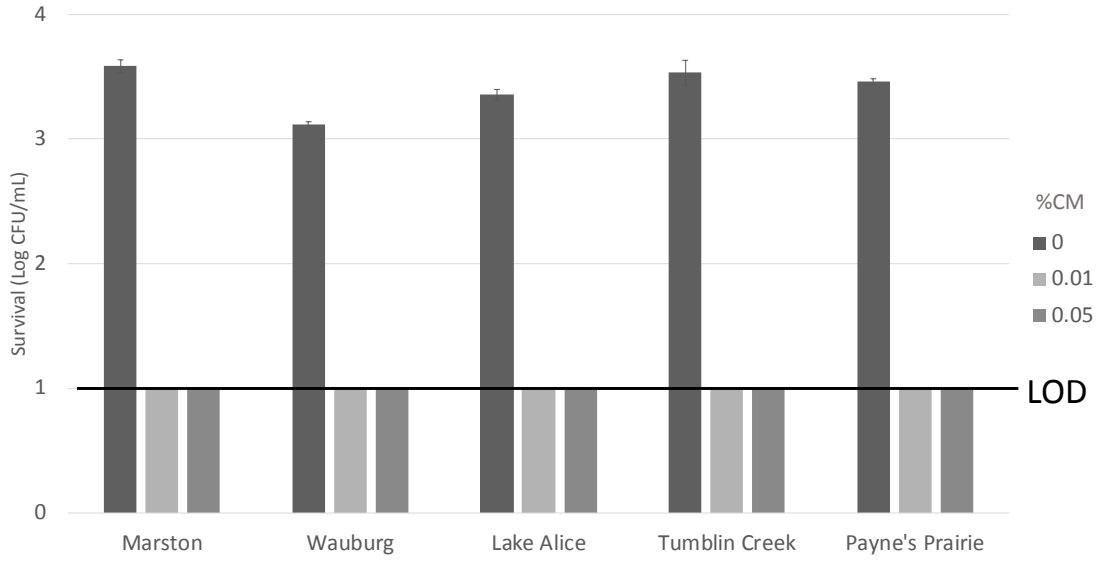

Figure 4. Effects of water source on anti-*Salmonella* activity of CM.
S. enterica Typhimurium 14028S was inoculated (ca. 3.7 log CFU/mL) into 250-mL flasks containing 50 mL of 0, 0.01, or 0.05% CMC. Solutions were prepared from water collected from different sources around Gainesville, FL, including Marston Pond, Lake Wauburg, Lake Alice, Tumblin Creek, and Paynes Prairie. Survival of *Salmonella* after 24 h was assessed by plate count on XLT4 agar. Error bars indicate standard error of the mean for three experiments. Limit of detection (LOD) was 10 CFU/mL.

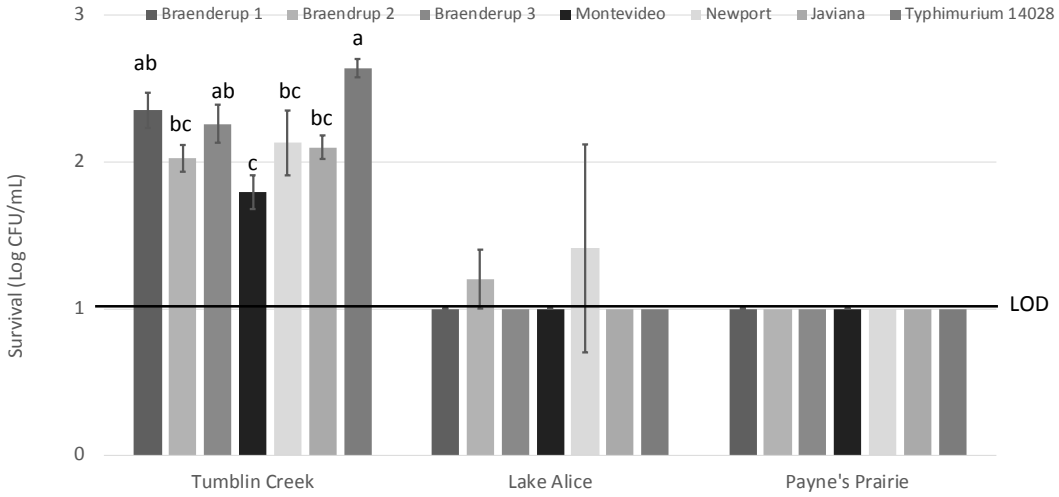

Figure 5. Effect of serotype on anti-*Salmonella* activity of CM in pond water from different sources. *S. enterica* serotypes Braenderup (1,2,3), Montevideo, Newport, Javiana, or Typhimurium 14028S were inoculated (ca. 3.0 CFU/mL) into 250-mL flasks containing 50 mL of 0, 0.01, or 0.05% CMC. Water sources included Tumblin Creek, Lake Alice, and Paynes Prairie in Gainesville, FL. Survival of *Salmonella* after 24 h was assessed by plate count on XLT4 agar. Limit of detection (LOD) was 10 CFU/mL.

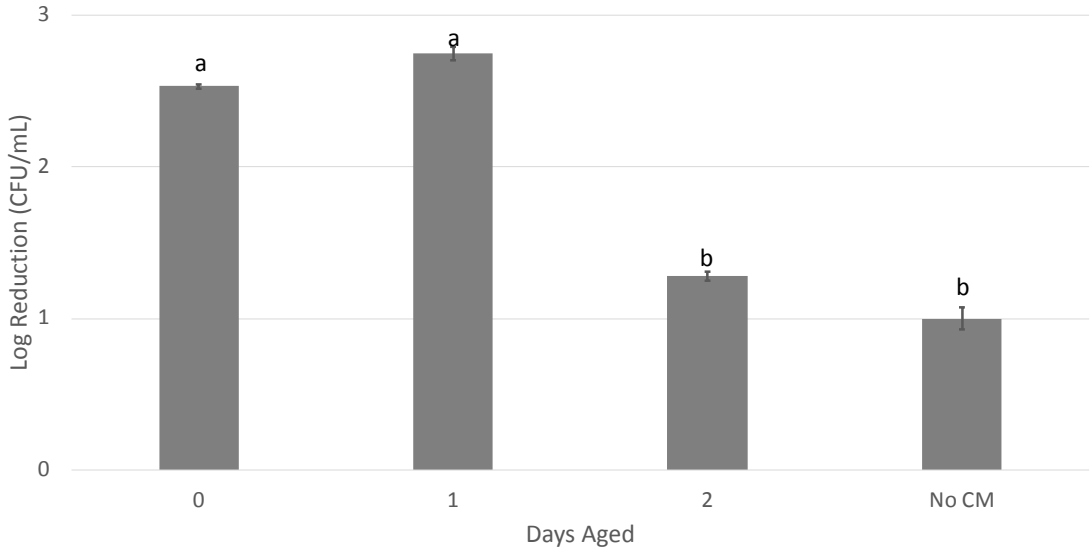

Figure 6. Stability of CM in pond water. CMC solutions were prepared in pond water and aged for 0, 1, and 3 days by incubation at room temperature. *S. enterica* Typhimurium 14028S was inoculated (ca. $5x10^3$ CFU/mL) into the CM solutions using 250-mL flasks containing 50 mL of 0.01% CMC. Log reduction of *Salmonella* after 24 h was assessed by plate count on XLT4 agar. Error bars represent standard error of the mean for three experiments.

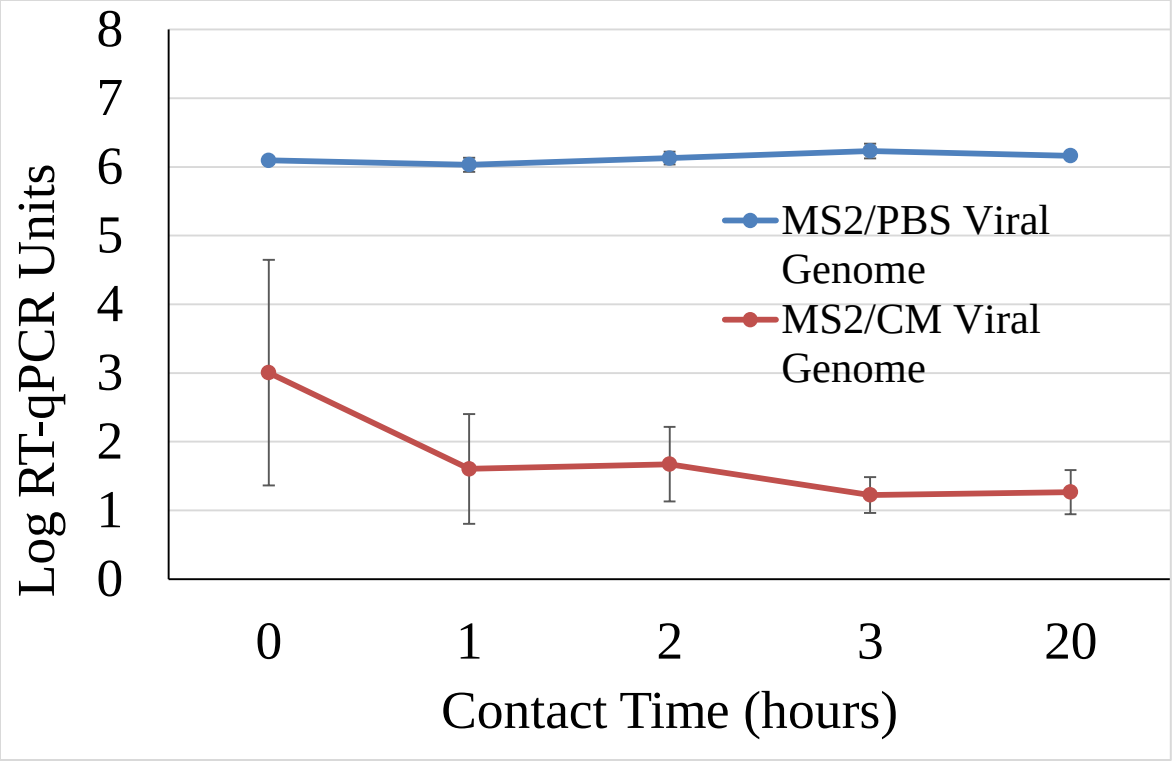

Figure 7. Reduction of MS2 virus following CM treatment (0.3%).

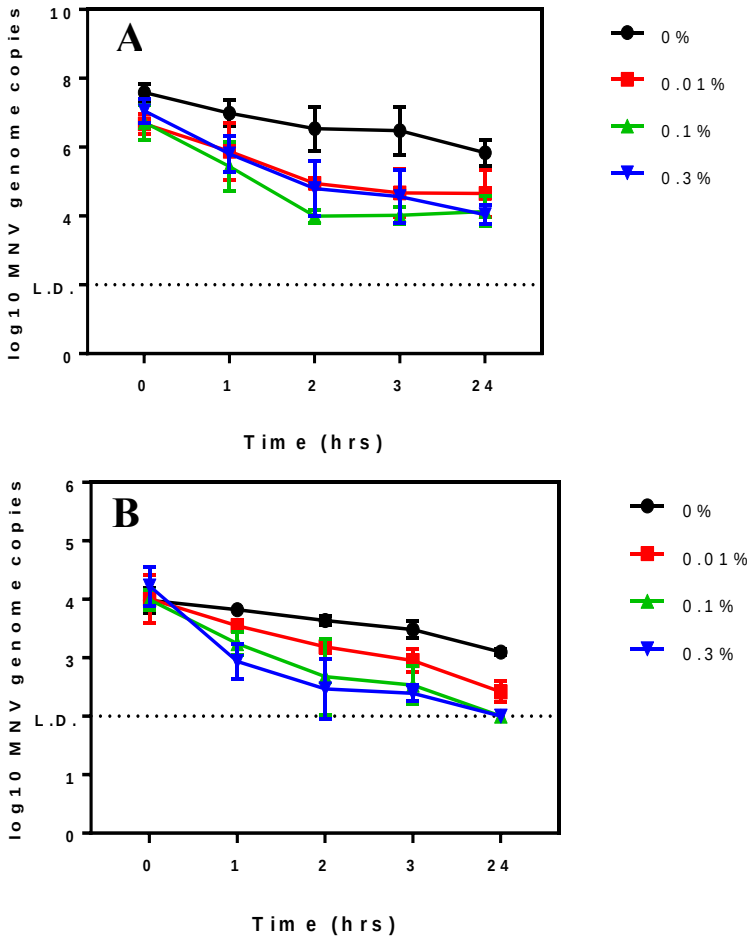

Figure 8. Reduction of murine norovirus following treatment with CM. a) Initial inoculum of 10^7 $\log_{10}$ $TCID_{50}$ MNV-1 and b) initial inoculum of 10^4 $\log_{10}$ $TCID_{50}$ MNV-1.

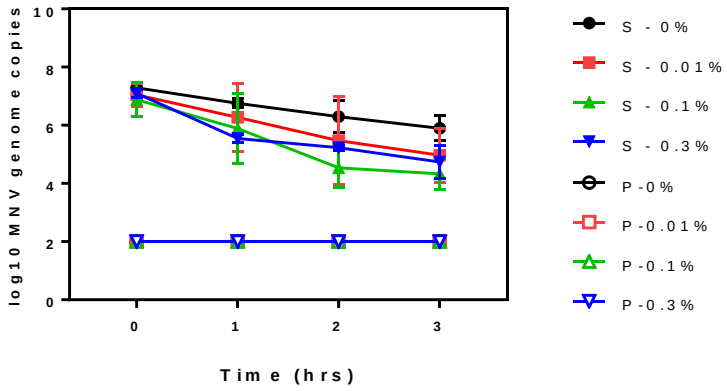

Figure 9. Murine norovirus does not bind to chitosan microparticles.

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