**Chitosan inhibits enterotoxigenic *Clostridium perfringens* type A in growth
medium and chicken meat**

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Running Title: Inactivation of *C. perfringens* by Chitosan.

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Abstract

Clostridium perfringens is a spore-forming bacterium and a major cause of bacterial food-borne illness. In this study, we evaluated the inhibitory effects of chitosan against spore germination, spore outgrowth and vegetative growth of *C. perfringens* food poisoning (FP) isolates. Chitosan of differing molecular weights inhibited germination of spores of all tested FP isolates in a KCl germinant solution containing 0.1 mg/ml chitosan at pH 4.5. However, higher level (0.25 mg/ml) of chitosan was required to effectively arrest outgrowth of the germinated *C. perfringens* spores in Trypticase-yeast extract-glucose (TGY) medium. Furthermore, chitosan (1.0 mg/ml) was bacteriostatic against vegetative cells of *C. perfringens* in TGY medium. Although chitosan showed strong inhibitory activities against *C. perfringens* in laboratory medium, higher levels (2.0 mg/g) were required to achieve similar inhibition of spores inoculated into chicken meat. In summary, the inhibitory effects of chitosan against *C. perfringens* FP isolates was concentration dependent, and no major difference was observed when using different molecule weight chitosan as an inhibitor. Our results contribute to a better understanding on the potential application of chitosan in cooked meat products to control *C. perfringens*-associated disease.

Introduction

Clostridium perfringens is a Gram-positive, anaerobic, spore-forming bacterium causing histotoxic and gastrointestinal (GI) diseases in humans and animals (McClane, 2007). *C. perfringens* type A producing *C. perfringens* enterotoxin (CPE) is the causative agent of *C. perfringens* type A food poisoning (FP), which currently ranks as the 2nd most commonly reported bacterial food-borne outbreaks in the United States (Hoffmann et al., 2012; Lynch et al., 2006; Scallan et al., 2011). This pathogenic bacterium is a concern to the food industry due to its ability to produce metabolically dormant spores that are highly resistant to various stresses related to food preservation approaches (Li and McClane, 2006a, 2006b; Paredes-Sabja et al., 2007; Sarker et al., 2000). Spore resistance properties facilitate the survival of FP isolates in processed meat and poultry, which are the products most commonly implicated in *C. perfringens* FP outbreaks (McClane 2007). When growth conditions in food items are favorable, these heat-resistant spores can germinate and outgrow into vegetative cells, reaching high viable cell numbers {~10⁶ colony forming Unit (CFU)/g} (McClane et al., 2013). After the food is consumed, viable cells that survived stomach acidity will sporulate in the GI tract, which leads to CPE release and subsequent GI illnesses (McClane 2007).

Currently, the food industry is interested in developing novel bacterial spore-inactivation strategies to meet the consumers' demand for natural, minimally processed and organic products. Alternative technologies include applications of natural antimicrobial agents in foods in order to maintain food safety, food quality, extended shelf life by controlling microbial spoilage and foodborne pathogen contamination. Chitosan is a natural carbohydrate polymer derived from the deacetylation of chitin, a main component of exoskeletons of crustaceans, insects, and the cell wall of fungi. Different production methods are used to produce chitosan with different chemical

properties, which potentially reflect variations in the antimicrobial activity (No and Meyers, 1995). Chitosan shows a broad-spectrum antimicrobial activity against both Gram-positive and Gram-negative bacteria, and fungi. Numerous factors may affect chitosan's antibacterial activities; most significantly among others are molecular weight (MW), degree of deacetylation (DD), physical state and pH (Kong et al., 2010). Most of the commercially available chitosan is shrimp-derived and is used as a food additive or preservative, and as an active packaging material (Friedman and Juneja, 2010; Kong et al., 2010; Su et al., 2009). It has been approved as a food additive in Korea's and Japan's generally-recognized-as-safe list (Kong et al., 2010). Intensive research has been focused on the application of different forms of chitosan including films, coatings, and nanochitosans, in different food categories (Friedman and Juneja, 2010; Su et al., 2009). However, information on its effect on bacterial spores, especially *C. perfringens* spores, and its possible application in poultry products is limited. Moreover, the fundamental knowledge of whether chitosan inhibits initiation of spore germination or prevents outgrowth of germinated spores remains unclear.

The objectives of this study were to examine the effects of chitosan against germination, outgrowth and vegetative growth of spores of the enterotoxigenic *C. perfringens* type A. Furthermore, the application of chitosan as an antimicrobial agent to control the germination and outgrowth of *C. perfringens* spores in cooked chicken meat during storage at extremely abusive temperature was also examined.

2. Material and methods

2.1. Bacterial strains and growth conditions

The *C. perfringens* isolates used in this study consisted of three type A FP isolates (SM101, NCTC10239, and E13) (Sarker et al., 2000). All stock cultures were maintained in cooked meat medium (Difco, BD Diagnostic Systems, Sparks, MD, USA) and stored at -20 °C. Bacteria were revived by inoculating the cooked meat stock culture into fluid thioglycollate (FTG) medium (Difco) and incubating overnight at 37 °C. TGY broth (3% trypticase, 2% glucose, 1% yeast extract, and 0.1% L-cysteine) was used for *C. perfringens* vegetative growth and spore outgrowth, as previously described (Paredes-Sabja et al., 2008b).

2.2. Spore preparation and purification

Spores of three *C. perfringens* type A strains (SM101, NCTC10239, and E13) were prepared and purified as described previously (Akhtar et al., 2008; Paredes-Sabja et al., 2008a). Briefly, the stock cultures of *C. perfringens* were inoculated into 10 ml FTG and incubated overnight at 37 °C. Then, 0.4 ml of an overnight FTG culture was inoculated into 10 ml FTG medium and incubated for 8 h at 37 °C. Next, 0.4 ml of 8-h FTG culture was inoculated into 10 ml Duncan-Strong sporulation medium (Duncan and Strong, 1968) and its sporulation capacity was confirmed under phase contrast microscope after incubation at 37 °C for 24 h. Large amounts of spores were prepared by scaling up the aforementioned procedure. Spores were purified by repeated washing and centrifuging with cold sterile distilled water until microscopic examination demonstrated that the suspensions were > 99% free of sporulating cells, cell debris, and germinated spores. Purified spores were suspended in sterile distilled water to obtain an optical density at 600 nm (OD₆₀₀) of ~ 6, and stored at -80 °C until use.

2.3. Preparation of chitosan solutions

Three stock solutions of chitosan of low MW (50-190 kDa), medium MW (190-310 kDa), and high MW (310-375 kDa), with 75-85% deacetylation, were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). Chitosan stock solutions (10 mg/ml) were prepared in 10 ml of 1% (v/v) acetic acid, according to the previously described protocol (Cruz-Romero et al., 2013; Li et al., 2010). These solutions were then filter sterilized (0.45 μ m, Millipore, Bedford, MA, USA) and stored at 4 °C. The stock solutions were used within a month of preparation. The stock solutions were then diluted as indicated in the results for each individual experiment.

2.4. *C. perfringens* spore germination in the presence of chitosan

The purified spore suspensions in water at a final OD₆₀₀ ~ 1.0 were heat activated at 80 °C for 10 min prior to germination as previously described (Paredes-Sabja et al., 2008b; Paredes-Sabja et al., 2009), and then cooled in a water bath at room temperature for 5 min. To examine the ability of chitosan to induce *C. perfringens* spore germination, heat-activated spores of strain SM101 were incubated with sterile distilled water as a control or with various concentrations (0.1, 0.5 or 1 mg/ml) of low molecular weight (LMW), medium molecular weight (MMW), or high molecular weight (HMW) chitosan (suspended in 1% acetic acid and adjusted to pH 6.0). To test the inhibitory effect of chitosan on spore germination, heat-activated spores of strain SM101 were incubated with pre-warmed 100 mM potassium chloride (KCl) in 25 mM Tris-HCl (pH 6.0) alone (control) or supplemented with (0.1 mg/ml) LMW, MMW, or HMW chitosan. To evaluate effect of pH on spore germination inhibition, the final pH of germinant solutions with or without chitosan were adjusted to the desired pH ranging from 3.5 to 6.5. Spore germination was routinely monitored by measuring OD₆₀₀ of the spore-germinant solutions (Smartspec 3000

spectrophotometer; Bio-Rad Laboratories, Hercules, CA), in which OD₆₀₀ is decreased by ~ 60% upon complete spore germination (Paredes-Sabja et al., 2008b). The extents of spore germination were also confirmed by phase-contrast microscopy in which germinated spores lose refractility and become phase dark. Germination rates were calculated as percentage loss of OD₆₀₀ relative to the initial value after 60 min of incubation in KCl with or without chitosan. Results were expressed as percent inhibition of spore germination compared with the control germination (without chitosan) as described previously (Alnoman et al., 2015; Cortezzo et al., 2004). To generalize our data, two additional *C. perfringens* type A FP strains (NCTC10239 and E13) were tested. All germination experiments were performed in triplicates with three different spore preparations for each strain.

2.5. Dipicolinic acid (DPA) release in the presence of Chitosan

The release of DPA during *C. perfringens* germination was performed as previously described (Paredes-Sabja et al., 2008b; Udompijitkul et al., 2014). Briefly, spores of FP strain SM101 (OD₆₀₀ of 1.5) were heat activated at 80 °C for 10 min, and then incubated with LMW chitosan (0.1 mg/ml), 100 mM KCl (control) or combination of 100 mM KCl and 0.1 mg/ml LMW chitosan, at 37 °C and at pH 4.5 and 6.0. One milliliter aliquots of germinating solutions were taken at various time periods and centrifuged for 3 min at 13,200 rpm in microcentrifuge tubes, and DPA in the supernatant fluids was determined by measuring absorbance at 270 nm (A_{270}). The DPA content in spore preparations was evaluated by boiling a 1-ml aliquot of germinating spores for 60 min, centrifuging for 5 min in a microcentrifuge, and measuring the A_{270} of the supernatant fluid. Our previous study indicated that ~90% of the material absorbing at 270 nm contained DPA in *C. perfringens* (Paredes-Sabja et al., 2008b).

2.6. *C. perfringens* spore outgrowth in the presence of chitosan

Three strains of *C. perfringens* type A FP (SM101, NCTC10239, and E13) were tested. Spore suspensions (0.3 ml) of an OD₆₀₀ of ~ 6.0 were heat activated at 80 °C for 10 min, and then cooled in a water bath at room temperature before inoculating into the pre-warmed 10 ml TGY broth (pH 6.0.) alone (control) or supplemented with LMW, MMW, or HMW chitosan (treatment) at different concentrations (0.05, 0.1 and 0.25 mg/ml). The highest concentration of chitosan tested caused the shift of the final pH of TGY medium by 0.2 unit. Cultures were incubated at 37 °C and the OD₆₀₀ was measured over time intervals up to 180 min post-inoculation. Spore outgrowth rates were calculated as percent increase in OD₆₀₀, and the percent inhibition of spore outgrowth was calculated as % inhibition = (OD₆₀₀ increase in treatment/ OD₆₀₀ increase in control) x100 (Alnoman et al., 2015; Cortezzo et al., 2004). The experiments were performed in triplicate with three independent spore preparations.

2.7. *C. perfringens* vegetative growth in the presence of chitosan

The inhibitory effects of chitosan on growth of *C. perfringens* FP strain SM101 were determined in conditions supporting vegetative growth in TGY broth. A 0.2-ml volume of an overnight FTG culture was transferred into 10 ml TGY and grown for 3 h at 37 °C. Then, 0.2 ml of the 3-h TGY culture was inoculated into fresh 10 ml TGY (pH 6.0) or supplemented with various concentrations of LMW chitosan ranging from 0.5 to 2.0 (mg/ml). Including the LMW chitosan at the highest concentration caused the final pH change of the medium from 6.0 to 5.0. A control sample of TGY without chitosan was included to compare the effects of chitosan. The growth was measured by enumerating colony forming unit (CFU)/ml of each treatment at 0, 3, 6 and 24 h post-inoculation. For CFU/ml counting, aliquots of cultures diluted in 25 mM Na₂HPO₄ (pH 7.5) buffer were plated onto Brain Heart Infusion (BHI) (Difco, BD Diagnostic Systems)

agar plates, incubated anaerobically at 37 °C for 24 h, and colony counted (Alnoman et al., 2015). All growth experiments were performed independently at least in triplicate and values are given as means.

2.8. Growth of *C. perfringens* spores in cooked chicken meat in the presence of chitosan

The ability of chitosan to prevent germination and outgrowth of *C. perfringens* spores in meat during storage at abusive conditions was measured as previously described (Alnoman et al., 2015; Udompijitkul et al., 2012). Briefly, grounded chicken meat samples (10g/bag) were placed into the UV-sterilized plastic bag (5.5" W × 6"L) and then sealed, autoclaved and stored at -20 °C until use. Prior to each experiment, bags containing meat sample were thawed and appropriate amounts (0, 0.5, 1.0, 2.0, 3.0 (mg/g)) of LMW chitosan stock solution were added. Then 0.1 ml of 3-strain spore cocktails were mixed by manually massaging for 1 min after resealing bags. The 3-strain spore cocktails of FP (SM101, NCTC10239, and FD1041) isolates were prepared by combining approximately equal number of spores from each strain containing ~10⁸ spores per ml. The negative control, sterile cooked chicken meat, was included in every replication to ensure that meat samples were free of naturally occurring bacteria. Samples were kept on ice before cooking at 80 °C for 15 min and cooled in water bath at room temperature for 15 min. For each treatment, one bag content was used to determine the initial population of *C. perfringens* in the contaminated meat, and another two samples were transferred to sterile petri dish and stored under anaerobic condition at 37 °C for 6 and 18 h. The pH of chicken samples without chitosan and with the highest tested concentration of chitosan (3 mg/ml) were 6.0 and 5.5, respectively. To determine populations of *C. perfringens* in the meat, each sample (10 g meat) was transferred into a stomacher bag, mixed with 90 ml 0.1% (w/v) peptone water, serially diluted in 25 mM

199 Na₂HPO₄ (pH 7.5) buffer, and then plated onto BHI agar plates that were incubated
200 anaerobically at 37 °C for 24 h before counting the colonies. Results were expressed as CFU/g.

201 ***2.9. Statistical analysis***

202 Data were analyzed by the analysis of variance procedure (ANOVA) using the statistical
203 software SAS version 9.3 (SAS Inst. Inc., Cary, N.C., USA). ANOVA among treatments was
204 performed and comparisons of mean values were established by Duncan's New Multiple Range
205 Test at the significant level of 0.05. In all figures, error bars represent the standard deviations.

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3. Results and discussion

3.1. Chitosan does not induce germination of spores of *C. perfringens*

When *C. perfringens* SM101 spores were incubated with 0.1 mg/ml of LMW, MMW, or HMW chitosan (suspended in 1% acetic acid and adjusted to pH 6.0) for 60 min at 37 °C, no change in OD₆₀₀ decrease, i.e., no germination was observed (Fig. 1). Phase contrast microscopy indicated that > 90 % spores were phase bright (data not shown). However, under similar experimental condition, SM101 spores germinated slightly with higher concentrations (0.5 and 1.0 mg/ml) of LMW, MMW, or HMW chitosan. Phase-contrast microscopy confirmed that only ~ < 20% spores became phase dark with higher concentrations of all tested chitosans (data not shown). These data indicate that chitosan, irrespective of MW, does not induce germination of spores of FP strain SM101 at pH 6.0.

3.2. Chitosan *inhibits* *C. perfringens* spore germination

As expected from our previous studies (Paredes-Sabja et al., 2008b), when SM101 spores were incubated with 100 mM KCl at pH 6.0, ~50% OD₆₀₀ decrease was observed (Fig. 2A), indicating significant germination. However, similar level of OD₆₀₀ decrease was observed with SM101 spores incubated with germinant solution of 100 mM KCl plus 0.1 mg/ml chitosan (LMW, MMW, or HMW) at final pH of 6.0, indicating no inhibition of spore germination (Fig. 2A). This is consistent with a previous study (Mellegard et al., 2011a) where no inhibition of *B. cereus* spore germination was observed with 0.5 mg/ml chitosan-HCl at pH 6.0.

As chitosan's activity is pH dependent (Kong et al., 2010), we examined the inhibitory effects of LMW chitosan (0.1 mg/ml) at different pHs ranging from 3.5 to 6.5. We found that inhibition of KCl-induced germination of SM101 spores was only achieved at pH 4.5 (data not

shown). Similar germination inhibition was observed with the addition of 0.1 mg/ml MMW or HMW chitosans at pH 4.5 (Fig 2B). Acidic pH alone was not the sole inhibitor for spore germination as SM101 spores successfully germinated with KCl adjusted to pH 4.5 to 6.5 (Fig. 2B and data not shown). Moreover, the presence of acetic acid, at the same concentrations used to dissolve chitosan (0.01% v/v) after dilution, exhibited an inhibitory effect on KCl-induced spore germination at pH 4.5 (Fig. 2B); nevertheless, the extent of germination inhibition was significantly less than those observed with chitosan incorporation, regardless of their MW. No significant difference ($p > 0.05$) on spore germination inhibition was observed with varying chitosan's MW (Fig. 2B).

When we extended germination inhibition experiments on three different FP strains including SM101 with 0.1 mg/ml of different MW chitosan, 89-99% inhibition of *C. perfringens* spore germination was observed with spores of all tested strains (Table 1). Further decreasing the concentration to 0.05 mg/ml inhibited spore germination up to 74-96% depending on strains and chitosan's MW (Table 1). Collectively, these data indicate that chitosan and acetic acid should have a synergistic effect on inhibition of KCl-induced spores germination of *C. perfringens* FP isolates at pH 4.5 regardless of MW of chitosans being assayed. Our results are consistent with an earlier study that reported a stronger antimicrobial activity of chitosan at pH 5.5 than at pH 6.5 against five species of foodborne pathogens including: *Staphylococcus aureus*, *Escherichia coli*, *Yersinia enterocolitica*, *Listeria monocytogenes*, and *Salmonella Typhimurium* (Wang, 1992).

3.3. Chitosan inhibits DPA release from spore core

To further validate the germination-inhibition capacity of chitosan, release of DPA from the spore core was monitored. Spores of various species of *Bacillus* and *Clostridium* generally

release DPA from the spore core during an early step of spore germination (Olguin-Araneda et al., 2015; Setlow, 2014). Therefore, we also examined the effects of chitosan on DPA release during KCl-induced germination of SM101 spores at pH 4.5 or 6.0. As expected, when SM101 spores were incubated with 100 mM KCl at 37°C for 60 min, most (~80-85%) of the spore core's DPA was released at pH 4.5 or 6.0 (Fig. 3), indicating complete spore germination. However, approximately 20 and 40% DPA was released when SM101 spores were incubated with chitosan (0.1 mg/ml LMW) at pH 4.5 and 6.0, respectively, indicating no significant germination as previously observed with OD₆₀₀ decrease (Fig. 1 and 3). However, while ~80% DPA-release was observed upon incubation of SM101 spores with 100 mM KCl supplemented with chitosan (0.1 mg/ml LMW) for 60 min at pH 6.0, DPA release decreased to ~ 45% when similar germination experiment was performed at pH 4.5 (Fig. 3). Collectively, these results indicate that chitosan (0.1 mg/ml LMW) can inhibit KCl-induced DPA release from SM101 spore core at pH 4.5 which provides further support that chitosan can inhibit KCl-induced germination of *C. perfringens* spores at pH 4.5. Our finding that *C. perfringens* SM101 spores released most DPA upon incubation with 100 mM KCl plus 0.1 mg/ml chitosan-acetate at pH 6.0 is consistent with similar data in the *B. cereus* system (Mellegard et al., 2011a).

3.4. Chitosan inhibits *C. perfringens* spore outgrowth

A previous study (Mellegard et al., 2011a) reported that chitosan (≤ 0.5 mg/ml) was able to inhibit *B. cereus* spore outgrowth when suspended in growth medium (pH 6.0) containing 25 mM L-alanine and 2.5 mM inosine as germinants. Therefore, the inhibitory effect of chitosan against *C. perfringens* spore outgrowth was assessed after inoculation of *C. perfringens* spores into rich growth medium. SM101 spores initiated outgrowth after incubation for ~ 60 min and 37°C in rich TGY medium (pH 6.0) (Fig. 4). However, the incorporation of 0.25 mg/ml LMW-,

MMW-, or HMW- chitosan into TGY significantly delayed SM101 spore outgrowth until ~120 min, and significant inhibition of spore outgrowth, as compared to the control, was observed after 180 min of incubation at 37°C (Fig. 4 and Table 2). Moreover, lower concentrations (0.05 and 0.1 mg/ml) of chitosan resulted in a slight inhibition of outgrowth rate as compared to the control. Unlike the effect of chitosan concentration, no significant effect of chitosan's MW was observed ($p > 0.05$) (Fig. 4 and Table 2). When similar outgrowth experiments were performed using spores of two additional FP strains (NCTC10239 and E13), slightly less % inhibition of spore outgrowth compared to SM101 was observed with all tested chitosans (Table 2), suggesting that inhibition of spore outgrowth by chitosan is strain specific. The observed lower extent of chitosan-induced inhibition of spore outgrowth compared to that of germination inhibition, especially in the case of HMW chitosan (Table 1 and 2) might be due to the lower susceptibility of the outgrowing spores to chitosan than germinating spores. Another possible explanation for this phenomenon is that chitosan might lose some antimicrobial activity due to competitive interaction of existing medium cations with negatively charged bacterial cell wall (Kong et al., 2010).

These data demonstrate that chitosan-induced inhibition of spore outgrowth depends on the concentration, but not the MW of chitosan (Table 2). However, previous studies with *B. cereus* spores suggested that chitosan's effect is dependent on fraction of acetylation and MW (Mellegard et al., 2011a). This discrepancy correlates with the previous study by Eaton et al. (2008), which suggested the strong dependency of chitosan's activity on target microorganisms and its MW (Eaton et al., 2008).

3.5. Chitosan inhibits the growth of *C. perfringens* vegetative cells

As our results showed that higher concentration of chitosan was required to inhibit spore outgrowth in TGY rich medium, we examined the vegetative growth of *C. perfringens* FP strain SM101 in TGY medium supplemented with various concentrations of chitosan. Results demonstrated that the incorporation of 0.5 mg/ml of chitosan to TGY gave log CFU/ml similar to that of control samples during the entire period of sampling (Fig. 5). However, increasing the chitosan level to 1 mg/ml in TGY strongly inhibited vegetative growth, as only a very slight elevation of log CFU/ml was observed after 24 h incubation. Interestingly, higher levels of chitosan (1.5 and 2.0 mg/ml) reduced the 3-h culture populations by ~ 2 log CFU/ml and their effects lasted up to 6 h of incubation. In contrast, longer incubation for up to 24 h resulted in 0 and 3 log CFU/ml reduction from initial counts in presence of 1.5 and 2.0 mg/ml LMW chitosan, respectively (Fig. 5). Phase-contrast microscopy revealed an aggregation of long filamentous cells in SM101 cultures grown in TGY supplemented with 0.5 mg/ml LMW chitosan (Fig. 6). Furthermore, severe aggregation and clumping of SM101 cells was observed when grown in TGY plus 1.0 mg/ml chitosan (data not shown). These cell deformation characteristics could correlate with the lack of increased cell numbers in TGY supplemented with higher concentrations of chitosan, because treatment with chitosan might be inhibiting cell division. Collectively, vegetative growth of *C. perfringens* was suppressed by LMW chitosan in a concentration-dependent manner; with 1.0 mg/ml LMW chitosan acts as a bacteriostatic agent and 2.0 mg/ml as a bactericidal agent. The MMW and HMW chitosan showed inhibition of *C. perfringens* vegetative growth similarly as LMW chitosan (data not shown). Similar results were found with two additional FP strains, E13 and NCTC10239 (data not shown).

This study revealed that the higher concentrations of chitosan were required to inhibit *C.*

perfringens vegetative cell growth relative to spore outgrowth which resembles the inhibitory effects of chitosan on *B. cereus* spore outgrowth and vegetative cells (Tsai et al., 2006). Similar to our findings, a previous study (No et al., 2002) reported the minimum inhibitory concentration (MIC) of chitosan to be >1 mg/ml against *B. cereus* and *E. coli*. However, noticeably lower MIC values of 0.1 and 0.003 mg/ml to control *B. cereus* and *E. coli*, respectively, were also reported (Mellegard et al., 2011b). These variations on MICs might presumably be linked to different characteristics of chitosan preparations used in each study (Mellegard et al., 2011b).

The precise mechanism of inhibition of chitosan towards *Clostridium* spp. has not been established. However, chitosan was reported to cause damage to the cell membrane of Gram-positive bacteria (Vårum and Smidsrød, 2005). In vegetative cells of *B. cereus* ATCC 14579, chitosan (MW 28.4 kDa and degree of acetylation 0.16) induced a significant efflux of potassium ion indicating an increase in cell membrane permeability and membrane damage (Mellegard et al., 2011b).

3.6. Chitosan blocks *C. perfringens* growth in cooked chicken meat products

To examine the inhibitory effects of chitosan on *C. perfringens* spore growth in cooked chicken meat during storage at abusive conditions, we employed our previously described meat model system (Akhtar et al., 2008; Alnoman et al., 2015; Udompijitkul et al., 2012). Our data (Fig. 7) demonstrated an increase of ~3 log CFU/ml viable cell count in *C. perfringens* spore-contaminated cooked chicken meat incubated anaerobically for 18 h at 37 °C. However, the presence of chitosan in cooked chicken meat at a concentration of 1 mg/g resulted in 1 log CFU/ml decrease in *C. perfringens* viable cell count (Fig. 7). Moreover, increasing the amount of chitosan in cooked chicken meat to ≥ 2 mg/g resulted in ~ 3 log CFU/ml reduction in *C. perfringens* viable cell count compared to the control sample after 18 h storage under anaerobic

condition at 37 °C (Fig. 7). Collectively, these data illustrate the inhibitory effect of chitosan against the growth of contaminating *C. perfringens* FP spores in meat samples under abusive storage conditions.

A previous study reported that a 3% (30 mg/g) solution of chitosan (degree of acetylation = 0.14) mixed into ground beef or turkey resulted in 4 to 5 log reductions of *C. perfringens* spore germination and outgrowth during exponential cooling of cooked beef or turkey up to 18 h (Juneja et al., 2006). However, in the current study, with 10-fold lower concentration of chitosan (3 mg/g) we obtained 3 log CFU/ml reductions in *C. perfringens* spore growth in cooked chicken meat under extremely abusive storage condition. One possible reason for this discrepancy might be the physical state and chemical characteristics of chitosan, as we used LMW chitosan solution dissolved in acetic acid; however, Juneja et. al. (Juneja et al., 2006) used chitosan-glutamate applied as a solid into meat products. Another possible reason might be food matrices; we used fat-free ground chicken, while Juneja et. al. (Juneja et al., 2006) used ground beef (25% fat) and turkey (7% fat).

Conclusions:

The present study demonstrated, for the first time, the inhibitory effects of chitosan against *C. perfringens* FP isolates in a variety of aspects including spore germination, outgrowth, and vegetative growth in laboratory medium and chicken meat model. At pH 6.0, all tested chitosans affected the outgrowth, but not the initiation of germination, of *C. perfringens* spores. However, significant inhibitions of spore germination and DPA release were observed with LMW-, MMW- or HMW-chitosan at pH 4.5. Since results obtained in this study revealed no significant differences in the inhibitory properties of three different MW chitosans against *C. perfringens*, the use of LMW chitosan, which also exhibited better solubility (Chang et al., 2015;

Friedman and Juneja, 2010; Tsai et al., 2006), was encouraged We also used LMW chitosan especially where higher concentrations were tested to avoid the high viscosity of chitosan solution. The LMW chitosan, albeit at higher concentration, showed bacteriostatic and bactericidal activity against vegetative cells of the enterotoxigenic *C. perfringens*. Despite the fact that the previous study showed the effectiveness of chitosan in controlling *C. perfringens* in beef and turkey, we were able to control growth of *C. perfringens* spores inoculated into cooked chicken meat stored in improper condition for an extended period by using relatively lower concentration of LMW chitosan. It is noteworthy that our meat challenge study was performed with a relatively high spore inoculum, and cooked meat being stored at extreme abusive conditions in order to simulate the worst-case scenario promoting the rapid growth from spores. Even under these extreme conditions, the tested chitosan was still quite effective in controlling spore germination and outgrowth in the cooked chicken meat despite lacking sporicidal effects. Therefore, the use of chitosan as an alternative food preservative to control growth of *C. perfringens* spores in cooked meat products should be considered. Further studies with different types of meats as well as under practical conditions typically applied in food industries are necessary to establish the effective use of chitosan for the safety of food products.

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Figure legends:

Fig. 1. Germination of *C. perfringens* spores with chitosan. Spores of *C. perfringens* FP strain SM101 were heat activated and incubated with different MW chitosans at concentrations of 0.1 mg/ml (black bars), 0.5 mg/ml (white bars), or 1 mg/ml (gray bars) at pH 6.0. Spore germination was measured as described in Material and methods. Error bars represent standard deviation from the mean of triplicate experiments with three independent spore preparations. LMW MMW, or HMW indicates low-, medium-, or high-MW chitosan, respectively.

Fig. 2. Inhibitory effects of chitosan on *C. perfringens* spore germination. Heat-activated spores of *C. perfringens* FP strain SM101 were incubated with 100 mM KCl alone adjusted to the desired pHs or 100 mM KCl supplemented with 0.01% acetic acid (chitosan's solvent) or different MW chitosan (0.1 mg/ml) at pH 6.0 (A) or pH 4.5 (B). Spore germination was measured as described in Material and methods. Error bars represent standard deviation from the mean of triplicate experiments with three independent spore preparations. LMW, MMW, or HMW indicates low-, medium- or high-MW chitosan, respectively.

Fig. 3. Effects of chitosan on DPA release during KCl-induced germination of *C. perfringens* spores. Heat-activated SM101 spores were germinated with 100 mM KCl (filled diamonds), 0.1 mg/ml LMW chitosan (filled triangles) or combination of both (filled squares) at pH 6.0 (A) or 4.5 (B). At various times as indicated, DPA release was measured as described in Material and methods. Error bars represent standard deviation from the mean of triplicate experiments with three independent spore preparations.

Fig. 4. Effects of chitosan on *C. perfringens* spore outgrowth. Spores of *C. perfringens* FP strain SM101 were heat activated and inoculated into TGY broth supplemented with LMW (A)-, MMW (B)- or HMW (C)-chitosan. Outgrowth of spores was measured as described in Material and methods. Symbols represent concentrations: diamonds, 0 mg/ml; circles, 0.05 mg/ml; triangles, 0.1 mg/ml; and cross marks, 0.25 mg/ml. Each data point represents an average of triplicate experiments with three independent spore preparations.

Fig. 5. Inhibitory effects of chitosan on *C. perfringens* vegetative cell growth. *C. perfringens* SM101 cells were inoculated into TGY (control) or TGY supplemented with various concentration of LMW chitosan as indicated and incubated at 37°C. Samples were collected after 0 h (black bars), 3 h (white bars), 6 h (gray bars), and 24 h (white bars with black diagonal stripes) incubation, serially diluted and plated onto BHI agar, and colony forming unit per ml (CFU/ml) determined. Error bars represent standard deviation from the mean of triplicate experiments performed independently.

Fig. 6. Cell morphology of *C. perfringens* SM101 grown in TGY medium supplemented with LMW chitosan. Phase-contrast photomicrographs of cells of SM101 grown for 24 h at 37°C in TGY medium alone (A) or TGY supplemented with 0.5 mg/ml LMW chitosan (B). Representative fields were photographed at 1000x magnification.

Fig. 7. Inhibitory effects of chitosan on growth of *C. perfringens* spores in cooked chicken meat products. Spores of 3-strain cocktail of *C. perfringens* FP isolates were inoculated into cooked chicken meat samples containing various concentrations of LMW chitosan as indicated

and CFU formed by survival spores were determined by plating onto BHI agar and incubated anaerobically at 37°C for 24 h. Black bars, initial CFU, white bars, viable CFU in cooked chicken samples after 6 h, and gray bars, after 18 h of anaerobic incubation at 37 °C. Error bars represent standard deviation from the mean of two independent experiments with two different spore preparations.

Table 1: Inhibition of *C. perfringens* spore germination by different MW chitosan at pH 4.5^a

Concentration (mg/ml)	Inhibition of germination (%) $\pm$ SD ^b								
	SM101			NCTC10239			E13		
	LMW ^c	MMW ^c	HMW ^c	LMW	MMW	HMW	LMW	MMW	HMW
0.01	10.1 $\pm$ 4.9 Da	14.1 $\pm$ 2.7 Ca	12.6 $\pm$ 3.8 Ca	10.7 $\pm$ 0.8 Da	12.1 $\pm$ 3.3 Da	13.3 $\pm$ 3.5 Da	22.3 $\pm$ 4.4 Cb	28.7 $\pm$ 2.9 Bab	32.4 $\pm$ 8.3 Ca
0.03	33.8 $\pm$ 2.5 Cab	49.7 $\pm$ 3.7 Ba	30.6 $\pm$ 1.2 Bb	31.3 $\pm$ 2.8 Ca	47.6 $\pm$ 7.0 Ca	40.2 $\pm$ 5.0 Ca	50.9 $\pm$ 2.4 Bb	89.4 $\pm$ 2.2 Aa	61.9 $\pm$ 1.8 Bb
0.05	74.1 $\pm$ 4.9 Ba	79.4 $\pm$ 3.2 Aa	66.8 $\pm$ 3.2 Aa	61.5 $\pm$ 6.1 Ba	78.3 $\pm$ 6.8 Ba	64.6 $\pm$ 3.2 Ba	96.3 $\pm$ 1.3 Aa	91.3 $\pm$ 2.8 Aa	88.9 $\pm$ 3.2 Aa
0.1	89.2 $\pm$ 3.2 Aa	89.1 $\pm$ 2.5 Aa	89.3 $\pm$ 2.2 Aa	94.3 $\pm$ 3.1 Aa	95.5 $\pm$ 0.7 Aa	94.4 $\pm$ 2.9 Aa	98.8 $\pm$ 0.6 Aa	98.8 $\pm$ 1.4 Aa	98.9 $\pm$ 0.6 Aa

^a spores were germinated with 100 mM KCl solution containing chitosan at various concentrations as indicated and percentage of inhibition of germination was calculated as described in Material and methods.

^b values are the average of triplicate experiments from three different spore preparation. SD indicates standard deviation from the mean. Values followed by the same uppercase letters within a column and by the same lowercase letters within a row for each isolate are not significantly different ($P > 0.05$).

^c LMW, MMW, HMW indicates low-, medium- or high-MW chitosan, respectively.

Table 2: Inhibition of *C. perfringens* spore outgrowth by different MW chitosan at pH 6.0^a

Concentration (mg/ml)	Inhibition of outgrowth (%) $\pm$ SD ^b								
	SM101			NCTC10239			E13		
	LMW ^c	MMW ^c	HMW ^c	LMW	MMW	HMW	LMW	MMW	HMW
0.05	47.3 $\pm$ 8.9 Ba	48.6 $\pm$ 15.6 Ba	40.5 $\pm$ 5.8 Ba	35.1 $\pm$ 7.7 Ba	31.5 $\pm$ 15.2 Bab	14.2 $\pm$ 18.9 Cb	22.4 $\pm$ 3.2 Ca	19.5 $\pm$ 3.9 Ca	6.3 $\pm$ 6.6 Cb
0.1	61.9 $\pm$ 10.6 Bab	72.5 $\pm$ 12.9 Aa	48.5 $\pm$ 8.1 Bb	61.9 $\pm$ 8.5 Aa	48.9 $\pm$ 3.2 ABab	37.2 $\pm$ 8.4 Bb	47.8 $\pm$ 6.2 Ba	41.5 $\pm$ 4.4 Ba	38.9 $\pm$ 1.7 Ba
0.25	82.3 $\pm$ 0.7 Aa	76.1 $\pm$ 3.8 Aab	71.7 $\pm$ 6.8 Ab	61.1 $\pm$ 10.9 Aa	66.2 $\pm$ 7.2 Aa	62.2 $\pm$ 11.4 Aa	67.3 $\pm$ 11.6 Aa	65.4 $\pm$ 12.7 Aa	65.9 $\pm$ 13 Aa

^a Heat-activated spores were inoculated into TGY medium supplemented with different MW chitosan at various concentrations as indicated. Spore outgrowth was assessed after 3 h of incubation at 37°C and inhibition of outgrowth was calculated as described in Material and methods.

^b values are the average of triplicate experiments from three different spore preparation. SD indicates standard deviations from the mean. Values followed by the same uppercase letters within a column and by the same lowercase letters within a row for each isolate are not significantly different ($P > 0.05$).

^c LMW, MMW, HMW indicates low-, medium- or high-MW chitosan, respectively.

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

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