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Chitosan Protects Cooked Ground Beef and Turkey Against *Clostridium perfringens* Spores During Chilling

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Chitosan Protects Cooked Ground Beef and Turkey Against *Clostridium perfringens* Spores During Chilling

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ABSTRACT: We investigated the inhibition of *Clostridium perfringens* spore germination and outgrowth by the biopolymer chitosan during abusive chilling of cooked ground beef (25% fat) and turkey (7% fat) obtained from a retail store. Chitosan was mixed into the thawed beef or turkey at concentrations of 0.5%, 1.0%, 2.0%, or 3.0% (w/w) along with a heat-activated 3-strain spore cocktail to obtain a final spore concentration of 2 to 3 $\log_{10}$ CFU/g. Samples (5 g) of the ground beef or turkey mixtures were then vacuum-packaged and cooked to 60 °C in 1 h in a temperature-controlled water bath. Thereafter, the products were cooled from 54.4 to 7.2 °C in 12, 15, 18, or 21 h, resulting in 4.21, 4.51, 5.03, and 4.70 $\log_{10}$ CFU/g increases, respectively, in *C. perfringens* populations in the ground beef control samples without chitosan. The corresponding increases for ground turkey were 5.27, 4.52, 5.11, and 5.38 $\log_{10}$ CFU/g. Addition of chitosan to beef or turkey resulted in concentration- and time-dependent inhibition in the *C. perfringens* spore germination and outgrowth. At 3%, chitosan reduced by 4 to 5 $\log_{10}$ CFU/g *C. perfringens* spore germination and outgrowth ($P \leq 0.05$) during exponential cooling of the cooked beef or turkey in 12, 15, or 18 h. The reduction was significantly lower ($P < 0.05$) at a chilling time of 21 h, about 2 $\log_{10}$ CFU/g, that is, 7.56 $\log_{10}$ CFU/g (unsupplemented) compared with 5.59 $\log_{10}$ CFU/g (3% chitosan). The results suggest that incorporation of 3% chitosan into ground beef or turkey may reduce the potential risk of *C. perfringens* spore germination and outgrowth during abusive cooling from 54.4 to 7.2 °C in 12, 15, or 18 h.

Keywords: beef, chilling, chitosan, *Clostridium perfringens*, growth inhibition, turkey

Introduction

Clostridium perfringens continues to remain a major cause of foodborne illness. Improper storage and/or inadequate cooling practices in retail food operations have been cited as a cause of food poisoning for 97% of *C. perfringens* outbreaks (Bean and Griffin 1990; Kalinowski and others 2003; Taormina and others 2003). According to the Centers for Disease Control and Prevention, the pathogen accounted for 3.2% cases of food poisoning and 2.1% of the outbreaks from 1993 to 1997 (CDC 2000). Foods associated with such outbreaks include cooked meat and poultry products (Bryan and McKinley 1979; Bryan 1988). Such foods are likely to be mishandled in an institutional food service setting, where these products may be prepared a day in advance and may be cooled at a slow rate or these products are not adequately refrigerated. These conditions favor germination and outgrowth of surviving, heat shocked *C. perfringens* spores into high numbers of vegetative cells. These cells are typically responsible for the reported illnesses. It is, therefore, important to cool and refrigerate foods quickly after cooking.

Products meeting the compliance guidelines for cooked, uncured, ready-to-eat (RTE) meat and poultry have not been linked to *C. perfringens* illness. The guidelines specify the cooling of cooked, uncured meat and poultry products from 54.4 to 26.7 °C in 1.5 h and from 26.7 to 4.4 °C within 5 h (preferred) or from 48.9 to 12.8 °C within 6 h (alternative), followed by additional cooling to 4.4 °C before packing (USDA/FSIS 2001). For cooked, cured meat and poultry products, the guidelines allow cooling from 54.4 to 26.7 °C in 5 h, and from 26.7 to 7.2 °C in 10 h. Cooling process deviations may occur in the food processing industry due to electrical outage or refrigeration equipment failure. In case of cooling deviations, USDA/FSIS requires that the relative growth of *C. perfringens* not exceed 1.0 $\log_{10}$ CFU/g during the cooling of certain meat and poultry products (USDA/FSIS 2001). In view of the potential hazards associated with the cooling of cooked foods, adding safe and effective antimicrobials to meat and poultry products can insure safety when the rate and extent of cooling of cooked products is not adequate.

Chitin, a component of the exoskeletons of insects and crustaceae including crab and shrimp, consists of *N*-acetylglucosamine residues joined by β (1-4) glycosidic links. It is the 2nd most abundant biopolymer in the world after cellulose. Structurally, chitin (poly-*N*-acetylglucosamine) resembles cellulose, except that the substituent at the carbon-2 atom is an acetylated amino (-NH-CO-CH_3) instead of a hydroxyl (OH) group. Deacetylation is achieved by exposing chitin to strong NaOH solutions or to the enzyme chitosinase (Figure 1). Interest in chitin resides in the fact that its

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deacetylated product called chitosan exhibits desirable functional and biological traits, including antimicrobial properties and the fact the biopolymer has received generally-accepted-as-safe (GRAS) status in the United States (Anonymous 2001).

Chitosan exhibits antimicrobial effects against a variety of pathogenic and spoilage organisms (see reviews by Rabea and others 2003; and Roller 2003). These include (a) spoilage and pathogenic microorganisms in meat (Darmadji and Izumimoto 1994); (b) spoilage microorganisms in chilled pork (Sagoo and others 2002); (c) waterborne coliform pathogens (Chen and others 2002); and (d) microorganisms adhered to stainless steel surfaces (Knowles and Roller 2001). Related studies on the antimicrobial effects of chitosan, chitosan derivatives, and chitosan films are described elsewhere (Ouattara and others 2000; Rhoades and Roller 2000; Jumaa and others 2002; Tsai and others 2002; Kim and others 2003; Nychas and others 2003; and Pranoto and others 2005). Generally, chitosan is more effective against gram-negative than against gram-positive bacteria (Devlieghere and others 2004). The polymer also protected mice against infection by *Listeria monocytogenes* (Okawa and others 2003), suggesting that the antimicrobial action may also occur in humans.

Because chitosan has not been previously evaluated for its ability to control *C. perfringens* in meat and poultry and because it is widely used as a food additive and in pharmaceutical formulations (Hirano 1997; Shepherd and others 1997; Shahidi and others 1999; Singla and Chawla 2001; Roller 2003), the major aim of this study was to explore its potential to control or inhibit outgrowth of *C. perfringens* from spores in ground beef and ground turkey during extended rate and extent of cooling after *sous vide* cooking.

Materials and Methods

Test compound

Chitosan glutamate, a deacetylated derivative of chitin, was obtained from Pronova Biopolymer (Drammen, Norway). This compound contained 42% glutamic acid and had 86% degree of deacetylation (information provided by the manufacturer).

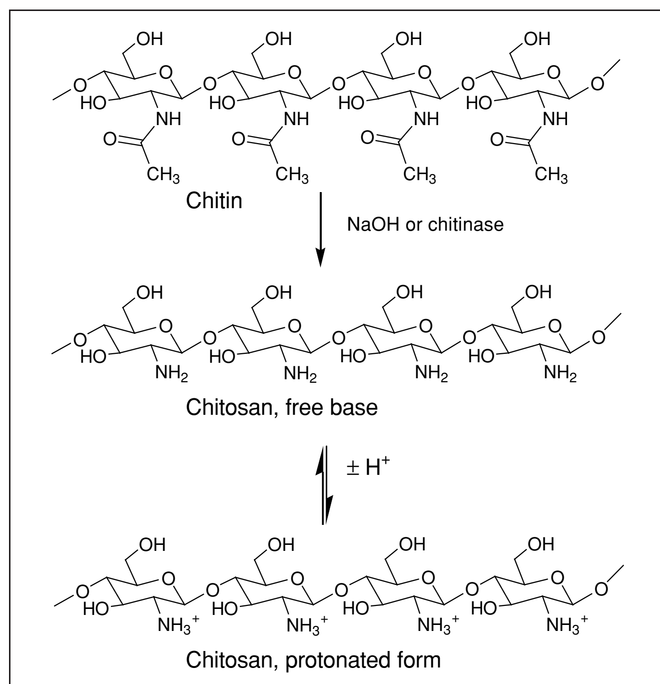


Figure 1 — Deacetylation of chitin to chitosan and acid-base equilibrium of chitosan. The antimicrobial effect of the free base is postulated to involve chelation to trace elements and metalloenzymes, and of the protonated form to disruption of cell membranes. See text.

Test organisms and spore production

Three strains of *C. perfringens*, NCTC 8238 (Hobbs serotype 2), NCTC 8239 (Hobbs serotype 3), and NCTC 10240 (Hobbs serotype 13), were obtained from the Microbial Food Safety Research Unit culture collection (Wyndmoor, Pa., U.S.A.). *C. perfringens* spores were produced in modified formulation of the Duncan and Strong sporulation medium as described previously (Juneja and others 1993). Briefly, the spore crop of each strain was washed twice with sterile distilled water and then resuspended in sterile distilled water. The suspensions were stored in a refrigerator at 4 °C. Spore population was enumerated by spiral plating (Spiral Biotech, Bethesda, Md. U.S.A.; Model D) of appropriate dilutions (in 0.1% peptone water), in duplicate, on tryptose-sulfite-cycloserine agar without cycloserine, that is, SFP agar (Difco, Detroit, Mich., U.S.A.) followed by incubation of plates anaerobically for 48 h at 35 °C. A spore cocktail was prepared immediately prior to experimentation by mixing equal numbers of spores of *C. perfringens* from each of the 3 suspensions. This composite of spore strains was heat-shocked for 20 min at 75 °C prior to use.

Preparation and inoculation of meat

Ground beef (25% fat) or turkey (7% fat) was obtained from a local retail market and frozen (−5 °C) until use (approximately 40 d). Chitosan was mixed into all ground beef or turkey samples with a Hobart mixer to final concentrations of 0.5%, 1.0%, 2.0%, or 3.0% (w/w). Duplicate ground beef or turkey (5-g samples) was then aseptically weighed into low-oxygen transmission Whirl Pak bags (4-oz/120-mL capacity; 3″W × 7 1/4″ L; 7.5 cm × 18.5 cm; barrier film 0.125 cc oxygen transmission per 100-in. square in 24 h [Part nr B01298WA; Nasco, Modesto, Calif., U.S.A.]) and inoculated with 1 mL of the heat-shocked *C. perfringens* spore cocktail to a final concentration of spores was approximately 3.0 $\log_{10}$ CFU/g. The bags were thoroughly mixed manually to ensure even distribution of the spores in the meat sample. Negative controls consisted of bags containing noninoculated ground beef or turkey. The bags were then evacuated to a negative pressure of 1000 millibars and heat sealed using a Multivac gas-packaging machine (Model A300/16, Multivac Inc., Kansas City, Mo., U.S.A.).

Cooking and cooling procedures

The bags containing inoculated products were sandwiched between stainless steel wire racks as described elsewhere (Thippareddi and others 2003) and submerged completely in a circulating water bath (Exacal, Model RTE-221, NESLAB Instruments, Inc., Newington, N.H., U.S.A.). The temperature of the water bath was increased to 60 °C in 1 h. The bags were then removed, chilled immediately in an ice water bath, and plated as described below. A 2nd set of racks containing the products for each treatment was cooked and transferred to the programmable water bath set at 54.5 °C. The bath equilibrated at this temperature for 10 min and then chilled at an exponential rate from 54.5 to 7.2 °C, according to the target chilling times depicted in Figure 2.

Enumeration of bacteria

Immediately after cooking and/or chilling, samples were removed and enumerated for total germinated *C. perfringens* population by spiral plating on tryptose-sulfite-cycloserine (TSC) agar as described previously (Juneja and Marmer 1998). The total *C. perfringens* population was determined after 48 h incubation at

37 °C in a Bactron anaerobic chamber (Bactron IV, Sheldon Laboratories, Cornelius, Oreg., U.S.A.). The lower limit of detection by this procedure is 21 CFU/mL. Both uninoculated raw beef and cooked beef (25 g) were used to verify the absence of naturally occurring *C. perfringens*. This variation involved the use of lactose-gelatin and nitrate-motility medium (Schwab and others 1984).

Statistical analyses

Two independent trials, each performed in duplicate, as defined by a new batch of meat, were performed for each of the exponential chilling rates (12, 15, 18, and 21 h). Data were analyzed by analysis of variance using the SAS General Linear Model procedure (SAS Inst., Inc., Cary, N.C., U.S.A.; Release 8.01). Bonferroni LSD method was used to separate means of the *C. perfringens* populations ($\log_{10}$ CFU/g) (Miller 1981).

Results and Discussion

Antimicrobial effectiveness of chitosan against *C. perfringens*

We investigated the inhibition of *C. perfringens* spore germination and outgrowth by the biopolymer chitosan during abusive chilling of cooked ground beef and turkey obtained from a retail store. Figure 2 shows the programmed and observed temperature profiles of the products for the 12, 15, 18, and 21 h exponential chill rates. The 4 temperature profiles represent extended chilling rates relative to the USDA/FSIS compliance guidelines for cooling of cooked, uncured meat and poultry products (USDA/FSIS 2001). The use of a water bath to generate the desired product temperature profiles is highly reproducible and can be used in future validation studies or for evaluation of microbiological safety of much larger quantities of meat products in meat processing operations.

Table 1 shows that chilling of ground beef resulted in germination and outgrowth of *C. perfringens* spores. Specifically, in the control samples without chitosan, cooling from 54.4 to 7.2 °C in 12, 15, 18, or 21 h, resulted in 4.21, 4.51, 5.03, and 4.70 $\log_{10}$ CFU/g increases, respectively, in *C. perfringens* populations of the ground beef. The corresponding increases for ground turkey were 5.27, 4.52, 5.11, and 5.38 $\log_{10}$ CFU/g.

Addition of chitosan to meat or turkey resulted in concentration- and time-dependent inhibition of growth of spores. At 3%, chitosan reduced by 4 to 5 $\log_{10}$ CFU/g *C. perfringens* spore germination and outgrowth during exponential cooling of the cooked beef in 12, 15, or 18 h ($P \leq 0.05$). The final *C. perfringens* population densities in

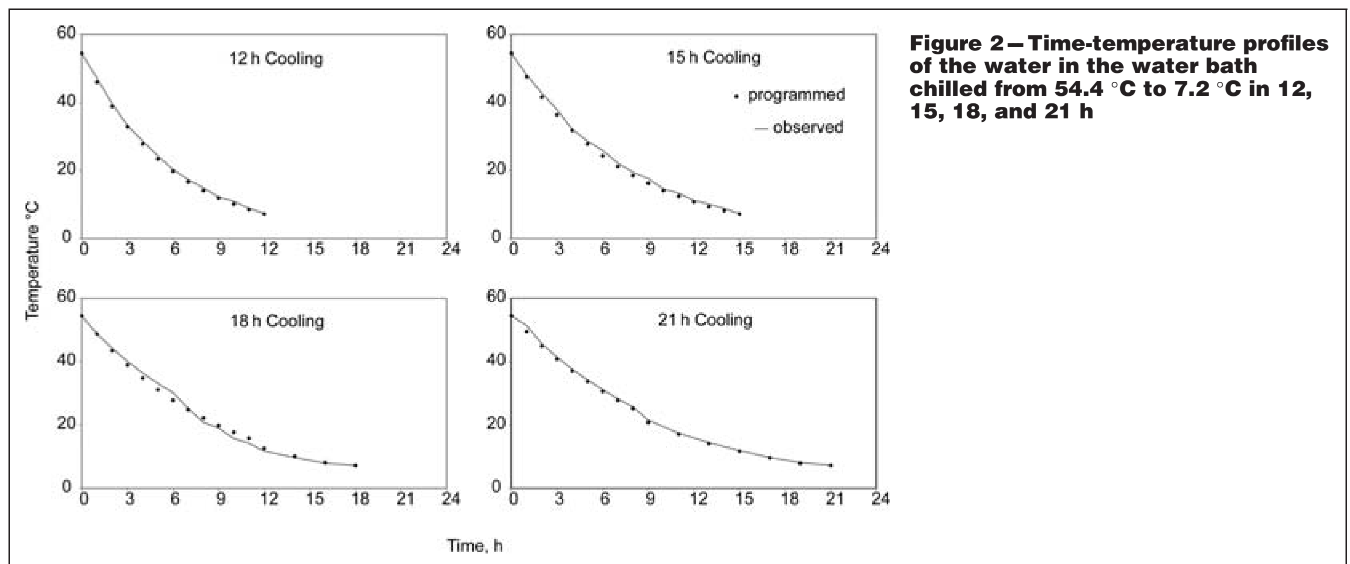


Figure 2 — Time-temperature profiles of the water in the water bath chilled from 54.4 °C to 7.2 °C in 12, 15, 18, and 21 h

Table 1 — Mean log CFU/g populations of *C. perfringens* in cooked ground beef and turkey containing chitosan immediately after heat treatment (cook; 60 °C in 1 h), and following cooling (chill) from 54.4 °C to 7.2 °C exponentially in 12, 15, 18, or 21 h

	12 h Chill		15 h Chill		18 h Chill		21 h Chill	
	Cook	Chill	Cook	Chill	Cook	Chill	Cook	Chill
Ground beef								
Control	2.14 (0.034)	6.35 (0.025)	3.29 (0.300)	7.80 (0.153)	3.23 (0.170)	8.26 (0.357)	2.86 (0.091)	7.56 (1.061)
Chitosan (%)								
0.5	2.23 (0.042)	4.31 (0.057)[b]	3.27 (0.121)	7.77 (0.033)	3.18 (0.016)	7.81 (0.089)	3.05 (0.044)	6.849).618)
1	2.43 (0.018)[a]	4.37 (1.269)[b]	3.47 (0.096)	7.10 (0.574)	3.20 (0.108)	7.87 (0.423)	2.82 (0.140)	7.21 (0.106)
2	2.72 (0.003)[a]	3.44 (0.000)[b]	3.50 (0.138)	5.59 (0.503)[b]	3.27 (0.097)	6.20 (0.404)[b]	3.06 (0.189)	6.02 (0.080)[b]
3	2.96 (0.069)[a]	3.22 (0.147)[b]	3.61 (0.061)	3.60 (0.108)[b]	3.47 (0.124)	3.61 (0.275)[b]	3.31 (0.069)	5.59 (0.392)[b]
Ground turkey								
Control	2.42 (0.432)	7.69 (0.067)	3.25 (0.190)	7.77 (0.266)	3.01 (0.224)	8.12 (0.330)	2.33 (0.494)	7.71 (0.776)
Chitosan (%)								
0.5	2.34 (0.007)	3.27 (0.269)[b]	2.93 (0.180)	7.55 (0.044)	2.80 (0.140)	7.80 (0.079)	2.49 (0.104)	6.46 (1.145)[b]
1	2.47 (0.021)	3.22 (0.358)[b]	3.25 (0.185)	6.39 (0.340)[b]	2.96 (0.279)	7.64 (0.232)	3.06 (0.240)	6.59 (0.806)[b]
2	2.74 (0.011)	2.96 (0.224)[b]	3.15 (0.053)	3.65 (0.254)[b]	3.20 (0.125)	5.08 (0.760)[b]	3.11 (0.250)	6.29 (0.808)[b]
3	2.49 (0.044)	2.91 (0.288)[b]	3.47 (0.017)	3.41 (0.137)[b]	3.41 (0.084)	3.26 (0.511)[b]	3.49 (0.080)[a]	5.14 (0.309)[b]

Values are averages from 2 separate experiments each in duplicate ± standard deviations shown in parentheses.
[a]Significantly greater than the corresponding control at $P < 0.05$ by Dunnett's test.
[b]Significantly less than the corresponding control at $P < 0.05$ by Dunnett's test.

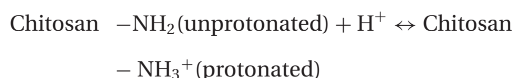
beef samples with added 3% chitosan after 12, 15, or 18 h exponential chill rates ranged from 3.22 to 3.61 $\log_{10}$ CFU/g. The reduction was significantly lower ($P < 0.05$) at a chilling time of 21 h, only about 2 $\log_{10}$ CFU/g, that is, 7.56 $\log_{10}$ CFU/g (unsupplemented) compared with 5.59 $\log_{10}$ CFU/g (3% chitosan). The results suggest that incorporation of 3% chitosan into ground beef or turkey may reduce the potential risk of *C. perfringens* spore germination and outgrowth during abusive chilling rates of 12, 15, or 18 h. The antimicrobial effects of adding corresponding amounts of chitosan to ground turkey were generally similar to those observed with ground beef (Table 1).

Table 1 also shows that for the beef and turkey "cook" data, 3 of the values for the 12 h chill and 1 value for the 21 h chill are higher than the corresponding controls. We have no apparent explanation for these results except to suggest that they may be due to chance and/or to the heterogeneity of the test substances.

Mechanisms of antimicrobial effects

The main mechanism that appears to govern the bacteristatic and bactericidal effects of chitosan appears to involve binding of its positively charged amino ($-NH_3^+$) groups (Figure 1) to negatively charged carboxylate ($-COO^-$) groups located on the surface of the bacterial cell membranes (Rabea and others 2003). Such electrochemical binding can alter the distribution of negative and positive charges on the surfaces of the cell membranes, leading to weakening and/or disruption of the membranes followed by leakage of cell components. This mechanism is supported by electron microscopy studies that showed that the polymer binds to and weakens the outer membrane of bacteria (Helander and others 2001) as well as by atomic force microscopy studies which indicate that chitosan nanoparticles induced disruption of cell membranes and leakage of cytoplasm of *Salmonella choleraesius* organisms (Qi and others 2004).

Because chitosan is also reported to strongly chelate toxic and essential metal ions (Friedman and Waiss 1972; Masri and others 1974; Piron and Domard 1998; Jeon and Park 2005), it is also likely that binding of chitosan to trace elements, such as ferric and zinc ions that the bacteria need for growth, may contribute to its antimicrobial action (Rabea and others 2003). The pH of the microenvironment in which chitosan operates determines the relative concentrations (ratios) of unprotonated and protonated amino groups which are governed by the equilibrium:



$$\text{Chitosan} \ -NH_2(\text{unprotonated}) + H^+ \leftrightarrow \text{Chitosan} - NH_3^+(\text{protonated})$$

The antimicrobial effectiveness of chitosan appears to be highest below pH 6 where the protonated form predominates and where chitosan is most soluble. By contrast, only the unprotonated form can chelate essential metal ions. These considerations suggest that depending on pH, different mechanisms may operate in different food categories and that lowering the internal pH of meat may enhance the antimicrobial activity of chitosan. The internal pH values of the ground meat and turkey used in this study were 6.25 and 6.46, respectively.

Conclusions

The results of the present study demonstrate for the 1st time that the widely used food additive chitosan adequately restricted, in a concentration-dependent manner, growth of *C. perfringens* spores in both ground meat and ground turkey up to a cooling time of 18 h ($P \leq 0.05$). The antimicrobial effectiveness was lower at the cooling time of 21 h than at the shorter time periods. These findings suggest that chitosan-containing meat and poultry products may benefit microbial food safety and the consumer. It would be of interest to find out whether low levels of chitosan in combination with low levels of organic acids and salts, which are reported at high concentrations to protect meat against *C. perfringens* (Juneja and Marmer 1998; Thippareddi and others 2003; Zaika 2003; Juneja and Thippareddi 2004; Sabah and others 2004), will exhibit additive or synergistic antimicrobial effects in meat and poultry matrices.

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