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Lethality enhancement of pressure-assisted thermal processing against *Bacillus amyloliquefaciens* spores in low-acid media using antimicrobial compounds

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ABSTRACT

Pressure-assisted thermal processing (PATP; 500–700 MPa, 90–121 °C) offers new opportunities to sterilize low-acid foods while preserving quality attributes to an extent greater than is possible with traditional thermal processing (TP). This study was conducted to evaluate the possibility of enhancing PATP lethality against the spores of *Bacillus amyloliquefaciens*, by sensitizing the spores with selected antimicrobial compounds (including emphasis on the use of natural antimicrobials) prior to treatment. A spore crop of *B. amyloliquefaciens* TMW 2.479 Fad 82, that had previously shown high resistance to combined pressure-heat treatment, was prepared on Nutrient Agar medium supplemented with 10 mg L^{-1} $MnSO_4 \cdot H_2O$ and incubated at 32 °C for 3 d. Spores were inoculated (at ~10^7–10^8 CFU mL^{-1} inoculum level) in HEPES buffer (pH ≤ 7.0) or selected low-acid foods (pH 5.2–5.6) with or without added antimicrobial compounds. The samples were then treated at 600 MPa and 105 °C (PATP) or 0.1 MPa and 105 °C (TP) for various holding times. Among different compounds tested, low-molecular-weight chitosan, and combination of chitosan with some surfactants were most effective ($P < 0.05$) in enhancing the PATP and TP lethality. This study suggests that certain antimicrobials can be added to the low-acid media prior to PATP or TP treatment to enhance the efficacy of the process. The treatment allows sterilization of low-acid foods at lower process temperatures thus ensuring better preservation of quality attributes.

1. Introduction

Most canned foods and other low-acid shelf-stable products are produced by thermal sterilization at temperatures above 100 °C to ensure the elimination of pathogenic and food spoilage bacterial spores. Thermal sterilization tends to adversely affect the product nutritional and quality attributes. Pressure-assisted thermal processing (PATP; 500–700 MPa, 90–121 °C) offers new opportunities to sterilize low-acid foods while preserving quality attributes to a greater extent than is possible with traditional thermal processing. The product heats rapidly and uniformly through adiabatic compression heating, and cools rapidly and uniformly upon decompression. This reduces product exposure to heat and allows better preservation of its quality, compared to conventional retorting (Rajan, Ahn, Balasubramaniam, & Yousef, 2006; Rajan, Pandrangi, Balasubramaniam, & Yousef, 2006). In 2009, the U.S. Food and Drug Administration (U.S. FDA) indicated no objection to an industrial petition for preserving low-acid shelf-stable foods using PATP technology (IFT News Releases, 2009). The petition primarily considered the lethal effects of heat without taking pressure lethality into consideration. Pressure was only used to increase the temperature up to 121 °C of a pre-heated product. This enables the industry to produce superior quality low-acid products such as soups, mashed potato, and other heat sensitive products. It is desirable to reduce further the thermal effect (to ≤105 °C, under pressure) before PATP becomes a commercially relevant option for preserving low-acid foods. An intermediate pathway for commercialization of pressure-heat-processed products has been processing low-acid foods by

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milder pressure-heat (60–90 °C under pressure) and distributing them under refrigeration (Daryaei, Balasubramaniam, & Legan, 2013; Van Opstal, Bagamboula, Vanmuysen, Wuytack, & Michiels, 2004).

Clostridium botulinum spores have been used traditionally to validate the safety of thermally-sterilized low-acid foods. However, the heat resistance of various strains of *C. botulinum* spores may not correlate with their resistance to combined high pressure-thermal treatment (Margosch, Ehrmann, Gänzle, & Vogel, 2004). A number of researchers have identified *Bacillus amyloliquefaciens* among bacteria that produce high pressure-thermal resistant spores comparable with those of *C. botulinum* (Ahn, Balasubramaniam, & Yousef, 2007; Hofstetter, Gebhardt, Ho, Gänzle, & McMullen, 2013; Margosch et al., 2006; NACMCF 2010; Rajan, Ahn et al., 2006). A 15-min PATP treatment at 700 MPa and 105 °C was required to inactivate *B. amyloliquefaciens* spores of TMW 2.479 Fad 82 strain in carrot purée to an undetectable level (~6.0 log reduction) with no regrowth during subsequent storage of the product at 32 °C (Ratphitagsanti, de Lamo-Castellví, Balasubramaniam, & Yousef, 2010). Addition of organic acid to the inoculated carrot purée prior to PATP treatment enabled treatment of the product for a shorter time (5 min) while inhibiting the spore recovery during storage (Ratphitagsanti et al., 2010).

Several researchers reported enhancement in antimicrobial efficacy of heat and high pressure using antimicrobial compounds. Although most of the compounds studied showed inhibitory effects (prevention of microbial outgrowth and recovery during storage), some including monolaurin and nisin and certain phenolic compounds from plant and fruit sources have been capable of enhancing process lethality against bacterial spores (Chaibi, Ababouch, Ghouila, & Busta, 1998; Gao & Ju, 2008; de Lamo-Castellví, Ratphitagsanti, Balasubramaniam, & Yousef, 2010; Komitopoulou, Boziaris, Davies, Delves-Broughton, & Adams, 1999; Shearer, Dunne, Sikes, & Hoover, 2000; Shin et al., 2008). This study was conducted to evaluate the possibility of enhancing PATP lethality against *B. amyloliquefaciens* spores by sensitizing the spores with selected antimicrobial compounds in a buffer system as well as in selected low-acid foods. A wide range of synthetic and natural antimicrobial compounds (including monoglycerides, benzoates and nitrites, chelating agents, surfactants, semi-synthetic polymers, antibiotics, bacteriocins, proteins, sucrose esters, phenolic compounds of essential oils, and plant and fruit extracts) were examined to find the most promising compounds resulting in the highest level of spore inactivation by PATP treatment.

2. Materials and methods

2.1. Spore culture strain

B. amyloliquefaciens TMW 2.479 Fad 82 culture was obtained from M. Gänzle (Department of Agricultural, Food and Nutritional Science, University of Alberta, Canada).

2.2. Spore preparation

B. amyloliquefaciens culture was initially grown aerobically in Trypticase Soy Broth supplemented with 0.6% yeast extract (TSBYE; Becton, Dickinson and Co., Sparks, MD, USA) at 32 °C for 24 h and then inoculated (0.5 mL) on Nutrient Agar supplemented with 0.6% yeast extract (NAYE; Becton, Dickinson and Co., Sparks, MD, USA) and 10 mg L^{-1} $MnSO_4{\cdot}H_2O$ (Fisher Scientific, Pittsburgh, PA, USA). The inoculated plates were then incubated at 32 °C for 3 d to obtain more than 90% sporulated population which was verified with a phase-contrast microscope. The spore crop was harvested by flooding the plates with cold sterile deionized water (DIW) and washed 5 times (14,000 × *g*, 10 min, 4 °C). The final spore suspension in sterile DIW (~10^9 CFU mL^{-1}) was heat-shocked (80 °C, 15 min) and kept at 4 °C until used.

2.3. Antimicrobial compounds

In a screening study, the lethal effect of combined PATP (600 MPa, 105 °C, 2 min) and antimicrobial compounds against *B. amyloliquefaciens* spores, suspended in 0.05 mol L^{-1} 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) buffer (pH 5.0–7.0), was investigated. The HEPES buffer used in this study was previously reported to exhibit minimal pH changes during high pressure treatment at ≤300 MPa and 20 °C compared to other buffer solutions including phosphate buffer and milk buffer (Molina-Gutierrez, Stippl, Delgado, Gänzle, & Vogel, 2002). A total of 35 different synthetic and natural antimicrobial compounds were tested at the concentrations given in Table 1. Most of the compounds tested in this study had previously shown inhibitory or lethal effects against vegetative cells or bacterial spores (Table 1). For these compounds, the selected concentrations were the highest concentrations tested by other researchers. The efficacy of the most promising antimicrobial compound or combination of compounds (with an emphasis on the use of natural antimicrobials) for enhancing the PATP lethality was then examined in selected low-acid foods including carrot purée (pH 5.2), squash purée (pH 5.4) and green bean purée (pH 5.6). The effects of compounds on spore inactivation by thermal processing (TP) at 105 °C and 0.1 MPa was also investigated in carrot purée.

The stock solution of each compound was freshly prepared in sterilized 0.05 mol L^{-1} HEPES buffer or DIW, except for nisin, chitosan and essential oil compounds which were prepared in 0.5% (v/v) acetic acid, 0.02 mol L^{-1} hydrochloric acid, and 1:1 water-ethanol solution, respectively. The solutions were filter-sterilized using a membrane filter (0.45 μm) before being added to the spore suspension in HEPES buffer or low-acid foods. The final pH was adjusted to the desired level using 10 mol L^{-1} sodium hydroxide.

2.4. Sample preparation for processing

To prepare the samples for processing, *B. amyloliquefaciens* spores were inoculated (~10^7–10^8 CFU mL^{-1}) in HEPES buffer or selected low-acid foods with or without added antimicrobial compounds in sterile polyethylene pouches (5 × 2.5 cm, sample size: 2 mL) for PATP, or in thermal-death-time (TDT) disks as described in section 2.6 (sample size: 1 mL) for thermal processing. The pouches or disks containing samples were kept at 4 °C for up to 2 h prior to treatment. Duplicate samples were treated in duplicate pressure-heat and thermal processing runs.

2.5. Pressure-assisted thermal processing treatment

A high pressure microbial kinetic tester (PT-1, Avure Technologies Inc., Kent, WA, USA), previously described in our publications (Nguyen, Rastogi, & Balasubramaniam, 2007; Ratphitagsanti et al. 2010) was used for pressure-heat treatment of spores in the media selected in this study. For combined pressure-heat treatment applications, the temperature of the external glycol bath was set at the desired target process temperature (105 °C) to minimize any heat loss during the test. The high pressure processor had a pressurization rate of about 20 MPa s^{-1}, while depressurization occurred within 2 s for all treatments. A 10-mL polypropylene syringe (model 309604, Becton, Dickinson and Company) wrapped with layers of tape (CVS® Pharmacy Inc., Woonsocket, RI, USA) was used as the sample holder. The temperature during treatment was monitored using procedure described by Ratphitagsanti et al. (2010). The syringe containing duplicate pouches was pre-heated

Table 1
List of selected antimicrobial compounds in the current study and their previously reported effects against microorganisms.

Groups	Compounds tested in this study and selected concentrations[a]	Previously reported effect	References
Monoglycerides	Monolaurin, monomyristin, monolinolein and monolinolenin (5 g L^{-1})	Inhibited outgrowth of *Bacillus cereus*, *Clostridium botulinum* and *Clostridium sporogenes* spores and vegetative cells in TSB or RCM broth (monomyristin and monolinolein); Enhanced thermal inactivation of *B. cereus* spores in pH 7.2 phosphate buffer (monolaurin); Inhibited outgrowth of *Bacillus* spp., *Geobacillus stearothermophilus, Alicyclobacillus* spp. and *C. sporogenes* spores in a model agar system (monolaurin); enhanced high-pressure inactivation of *B. subtilis* spores in milk (monolaurin)	Chaibi, Ababouch, and Busta (1996); Chaibi et al. (1998); Shearer et al. (2000)
Preservative salts	Sodium benzoate (0.5 g L^{-1}) and sodium nitrite (2 g L^{-1})	Inhibited germination and outgrowth of *Alicyclobacillus acidoterrestris* spores in malt extract broth (sodium benzoate)	Bevilacqua, Corbo, & Sinigaglia (2008)
Chelating agents	Sodium ethylenediaminetetraacetic acid (Na-EDTA) (2 g L^{-1})	Enhanced high-pressure inactivation of *Clostridium* spp. spores in roast beef; Enhanced thermal inactivation of *B. cereus*	Kalchayanand et al. (2003); Yang et al. (2011)
Synthetic anionic surfactants	Sodium dodecyl sulfate (SDS) (2 g L^{-1})	Inactivated yeasts (*Candida albicans*) by inducing cell lysis and release of vital intracellular components from the cells	Vieira and Carmona-Ribeiro (2006)
Semi-synthetic polymers	Low-molecular-weight chitosan (2 g L^{-1})	Inhibited germination of *A. acidoterrestris* spores in tomato juice; Inhibited germination and outgrowth of *Clostridium perfringens* spores in cooked ground beef and turkey during chilling; Enhanced thermal inactivation of *B. cereus*; Inhibited outgrowth of *Listeria monocytogenes* in bovine meat pâté	Falcone et al. (2005); Juneja et al. (2006); Bevilacqua, Sinigaglia, & Corbo (2008); Shin et al. (2008); Bento et al. (2011)
Antibiotics	Tylosin solution (5 ml L^{-1})	Used as an adjuvant in thermal processing (canning) to prevent the growth of *C. botulinum* and formation of botulism toxin	Chichester and Tanner (1972)
Bacteriocins	Pediocin (5000 IU/ml) and nisin (2000 IU mL^{-1})	Inhibited outgrowth of *Bacillus coagulans* spores high-pressure-treated in tomato juice (nisin); Enhanced thermal inactivation of *A. acidoterrestris* spores in fruit juices (nisin); Enhanced high-pressure inactivation and controlled outgrowth of *Clostridium* spp. spores in roast beef (pediocin combined with nisin); Enhanced high-pressure inactivation of *C. botulinum* spores in UHT milk (nisin); Enhanced thermal and pressure-thermal inactivation of *Clostridium* spp. and *Bacillus amyloliquefaciens* spores in a 0.9% saline solution (nisin)	Roberts and Hoover (1996); Komitopolou et al. (1999); Kalchayanand et al. (2003); Kalchayanand, Dunne, Sikes, and Ray (2004); Gao and Ju (2008); Hofstetter et al. (2013)
Proteins	Lactoferrin (0.5 g L^{-1}) and lysozyme (2 g L^{-1})	Enhanced high-pressure inactivation of *Shigella sonnei*, *Pseudomonas fluorescens* and *Staphylococcus aureus* (lactoferrin) in pH 7.0 phosphate buffer; Inactivated *A. acidoterrestris* spores and vegetative cells in saline solution, laboratory medium and apple juice (lysozyme)	Masschalck, Van Houdt, & Michiels (2001a,b); Masschalck, Van Houdt, Van Havre, & Michiels (2001); Conte et al. (2006); Bevilacqua et al. (2007); Bevilacqua, Sinigaglia, et al. (2008); Shin et al. (2008)
Semi-synthetic non-ionic surfactants	Sucrose esters, including sucrose stearate, sucrose palmitate and sucrose laurate (5 g L^{-1})	Inhibited outgrowth of *Bacillus* spp., *G. stearothermophilus*, *Alicyclobacillus* spp. and *C. sporogenes* spores in a model agar system (sucrose laurate); Enhanced lethality of pressure-assisted thermal processing against *B. amyloliquefaciens* spores in deionized water and mashed carrots (sucrose laurate)	Shearer et al. (2000); de Lamo-Castellví et al. (2010)
Phenolic compounds of essential oils	Eugenol, cinnamaldehyde, limonene, carvacrol, and thymol (0.5 g L^{-1})	Enhanced high-pressure inactivation of *Saccharomyces cerevisiae* vegetative cells in sterile distilled water and sodium chloride solution containing Tween 80 (limonene); Inhibited germination and outgrowth of *Bacillus megaterium* in nutrient broth (carvacrol and thymol); Inhibited germination and outgrowth of *A. acidoterrestris* spores in malt extract broth (eugenol and cinnamaldehyde)	Adegoke, Iwahashi, and Komatsu (1997); Periago, Conesa, Delgado, Fernández, and Palop (2006); Bevilacqua, Corbo, et al. (2008)
Plant and fruit extracts	Grapefruit seed extract, *Echinacea* extract, tea tree oil, turmeric, garlic purée, garlic extract, wasabi, mustard, mustard oil, green tea extract, ginseng, Japanese apricot extract, licorice extract, sarsaparilla extract, and yucca extracts (20 g L^{-1})	Enhanced thermal inactivation of *B. cereus* (grapefruit seed extract); Exhibited inhibitory or lethal effect against bacteria (*Escherichia coli* and *S. aureus*) and yeasts (*C. albicans*) by inhibiting respiration and increasing the permeability of bacterial cytoplasmic and yeast plasma membranes (tea tree oil)	Cox et al. (2000); Shin et al. (2008); Yang et al. (2011)

[a] Most of the compounds were tested at pH levels as high as 7.0 (buffer), except chitosan which was insoluble at pH above 6.0.

to an empirically-determined (Nguyen et al., 2007) pre-processing temperature (T_1, Table 2) in a water bath (Isotemp 928, Fisher Scientific) for 2 min. The syringe was then immediately loaded into the pressure chamber. The pressurization started after reaching a predetermined temperature, T_2 (Table 2), which was estimated using the following equation (Eq. (1)) where T_3, CH and ΔP are the desired target temperature (°C), heat of compression value of water (°C/100 MPa), and process pressure (MPa), respectively. ΔT_H is the temperature gained by the test sample during loading within the pressure chamber as well as pressurization (Nguyen et al., 2007):

$$T_2 = T_3 - (CH \cdot \Delta P + \Delta T_H) \quad (1)$$

The temperature of samples reached the estimated pre-process temperature within 3 min after preheating the samples in a water bath and subsequently loading into the pressure chamber (Table 2). The time required to reach the target pressure of 600 MPa was about 30 s. The pressure holding time did not include the pressure come-up time or the depressurization time. After depressurization, samples were immediately removed from the pressure chamber and cooled in an ice-water bath (4 °C) to prevent further spore inactivation. The samples were enumerated as described in a later section within 3 h after the treatments. All experiments were conducted in two independent pressure-heat treatment trials using duplicate samples.

2.6. Thermal processing

Thermal processing (TP) experiments were carried out on a selected low-acid food (carrot purée) at 105 °C and 0.1 MPa using 35-L circulating oil baths (*NESLAB EX-35 Digital One*, Thermo Fisher Scientific Inc., Waltham, MA, USA). Custom-fabricated TDT disks (18 mm diameter, 4.5 mm height) were used as sample holders (Jin, Zhang, Boyd, & Tang, 2008). The sample temperature was monitored and recorded as described by Ratphitagsanti et al. (2010). Two oil baths were used to manipulate thermal pre-processing time to that of pressure-heat treatment (Nguyen et al., 2007). Temperature of the first oil bath was maintained at 113 ± 1 °C. Once the sample temperature reached 100 °C, all TDT disks were shifted to the second oil bath which was maintained at the desired target process temperature (i.e. 105 °C). At specific holding times, the disks were removed from the second oil bath and immediately immersed into an ice-water bath (4 °C) to avoid further spore inactivation. Temperature histories were automatically recorded by the data logger. The surviving spore populations were enumerated within 3 h after the treatments. All experiments were carried out in two independent trials using duplicate samples.

2.7. Enumeration of survivors

Surviving *B. amyloliquefaciens* spores in HEPES buffer or foods subjected to pressure-heat treatment and thermal processing with or without addition of antimicrobial compounds were enumerated on TSAYE using the spread-plate method. The plates were incubated at 32 °C for 2 d before enumeration of survivors. The detection limit of the method was 10 CFU mL^{-1}.

2.8. Statistical analysis of data

Statistical analysis was performed by applying analysis of variance (ANOVA) and multiple comparisons of means of each treatment (with or without added antimicrobials) using Fisher's Least Significant Difference (LSD) test at the confidence level of 95%. Analysis of data was carried out with Statistical Analysis System software (SAS 9.2, SAS Institute Inc., Cary, NC, USA) and the difference between mean values greater than the $LSD_{(0.95)}$ was considered to be significant.

3. Results and discussion

As expected, application of antimicrobial compounds alone had no effect on *B. amyloliquefaciens* spores in our study (data not shown). The combined effects of antimicrobials and PATP or TP on spores are presented and discussed below.

3.1. Combined effects of antimicrobial compounds and PATP on spore inactivation in HEPES buffer

Figures 1 to 3 present the efficacy of combined PATP (600 MPa, 105 °C) and selected antimicrobial compounds, used individually or in combinations, on the inactivation of *B. amyloliquefaciens* spores in 0.05 mol L^{-1} HEPES buffer in the pH rage of 5.0–7.0. The antimicrobials listed in Table 1, but not included in these figures (such as monoglycerides and bacteriocins) had no effects on the PATP lethality against *B. amyloliquefaciens* spores under our experimental conditions. As presented in Table 1, some of these compounds were previously reported to be effective against other bacterial spores treated with thermal processing or high pressure processing at moderate process temperatures. For example, Chaibi et al. (1998) reported a significant reduction in the heat resistance of *Bacillus cereus* spores treated at 91–97 °C (0.1 MPa) in phosphate buffer in the presence of 0.005 mol L^{-1} monolaurin. Shearer et al. (2000) also reported a 3.0-log reduction of *Bacillus subtilis* spores in milk treated at 392 MPa and 45 °C for 10 min in the presence of monolaurin added at a concentration as low as 0.001%. No significant spore inactivation ($P > 0.05$) was achieved by a 2-min PATP treatment in the absence of antimicrobial compounds at pH 7.0 (only a 0.4 log reduction) (Fig. 1A). Addition of lysozyme at a concentration of 2 g L^{-1} (pH 7.0), prior to PATP treatment enhanced the process lethality against spores by nearly 1.0 log ($P < 0.05$) (Fig. 1A). The antimicrobial effectiveness of lysozyme against vegetative cells and bacterial spores has been reported by a number of researchers (Bevilacqua, Corbo, Buonocore, Del Nobile, & Sinigaglia, 2007; Conte, Buonocore, Bevilacqua, Sinigaglia, & Del Nobile, 2006;

Table 2
Temperature histories of spore samples suspended in HEPES buffer during pressure-heat treatment (600 MPa and 105 °C).

Holding time (min)	Pressure come-up time[a] (min)	Pre-process temperature[a] (T_1, °C)	Temperature immediately before pressurization[a] (T_2, °C)	Temperature immediately after pressure come-up time[a] (T_3, °C)	Temperature during holding time[a] (T_3 ~ T_4, °C)	Temperature immediately after depressurization[a] (T_5, °C)
0	0.5 ± 0.05	64 ± 0.5	68 ± 0.1	103 ± 0.1	105 ± 0.5	82 ± 0.6
1	0.5 ± 0.04	63 ± 1.0	66 ± 0.2	104.5 ± 0.6	106 ± 0.4	81 ± 0.5
2	0.5 ± 0.03	62.5 ± 0.3	65 ± 0.1	104 ± 0.7	106.5 ± 1.1	83.5 ± 0.2
3	0.5 ± 0.02	63 ± 1.0	66 ± 0.4	103 ± 0.2	105 ± 0.5	85 ± 0.7
5	0.5 ± 0.05	62 ± 0.2	69 ± 0.2	102 ± 0.5	105.5 ± 0.3	84 ± 0.7
8	0.5 ± 0.05	62 ± 0.2	67.5 ± 0.2	102 ± 0.5	106 ± 0.3	84 ± 0.7

[a] Data presented are means ± standard deviation of two independent pressure-heat treatment trials.

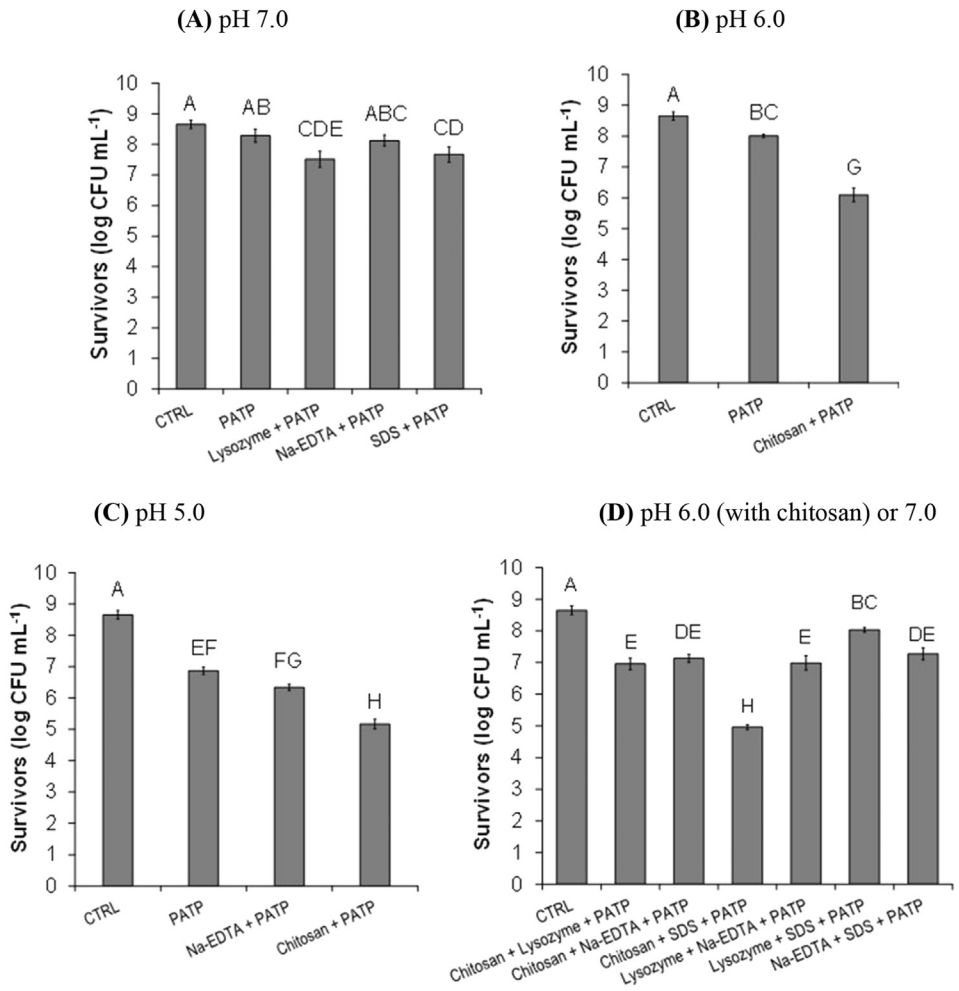

Fig. 1. Inactivation of *Bacillus amyloliquefaciens* spores by pressure-assisted thermal processing (600 MPa, 105 °C, 2 min) in 0.05 mol L^{-1} HEPES buffer at different pH levels without adding antimicrobials, and with addition of selected antimicrobial compounds used individually (2 g L^{-1} or 0.2%) or in combination (0.2% + 0.2%). The initial inoculum level was ~4.7 × 10^8 CFU mL^{-1}. Bar graphs and error bars represent the mean survivors (n = 4) and standard deviations, respectively. The means sharing a letter are not significantly different ($P > 0.05$). Controls represent untreated samples.

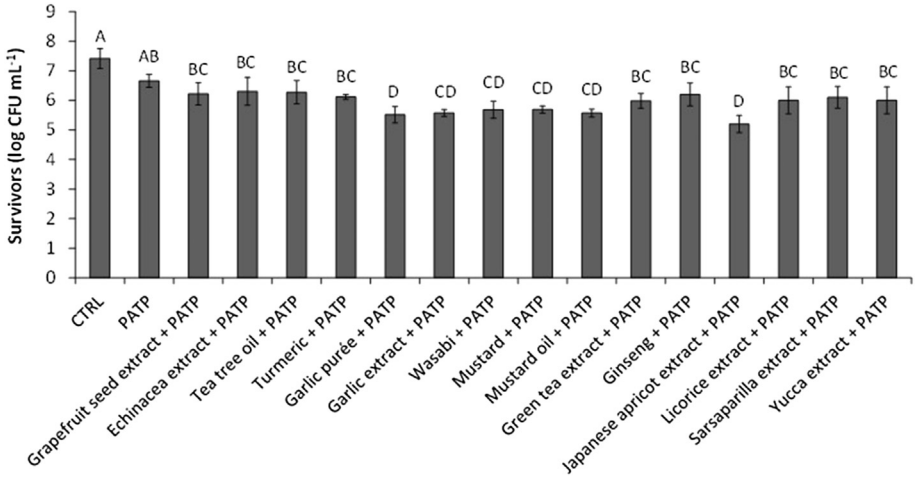

Fig. 2. Inactivation of *Bacillus amyloliquefaciens* spores by pressure-assisted thermal processing (600 MPa, 105 °C, 2 min) in 0.05 mol L^{-1} HEPES buffer (pH 6.8) without adding antimicrobials, and with addition of selected commercial natural antimicrobial compounds extracted from plant and fruit sources (2%). The initial inoculum level was ~3.4 × 10^7 CFU mL^{-1}. Bar graphs and error bars represent the mean survivors (n = 4) and standard deviations, respectively. The means sharing a letter are not significantly different ($P > 0.05$).

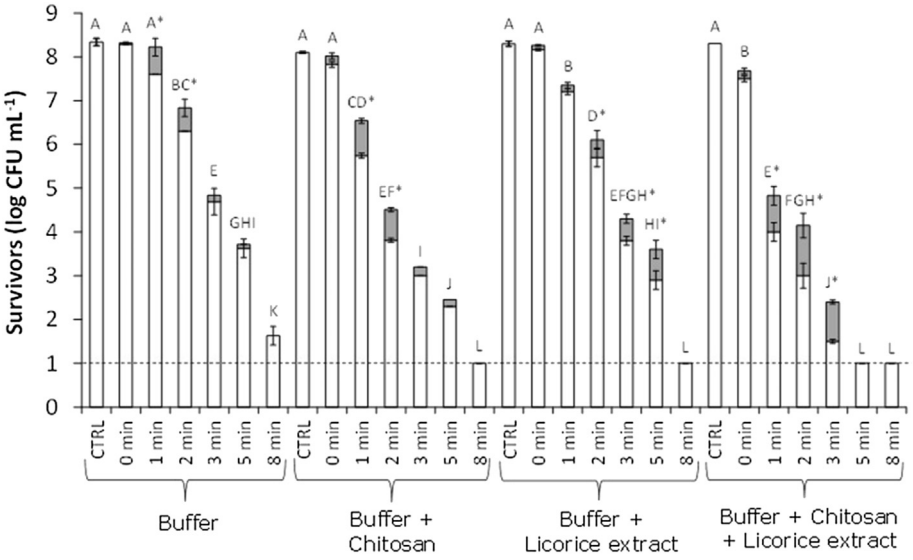

Fig. 3. Inactivation of *Bacillus amyloliquefaciens* spores by pressure-assisted thermal processing (600 MPa and 105 °C for 2 min) in 0.05 mol L^{-1} HEPES buffer (pH 6.0) without adding antimicrobials, and with addition of chitosan (0.2%), licorice extract (2%), or combination of chitosan and licorice extract (0.2% + 2%). The initial inoculum level was ~2.0 × 10^8 CFU mL^{-1}. Bar graphs and error bars represent the mean viable counts (n = 4) and standard deviations, respectively. (□) Dormant spores and (■) germinated spores. The dormant spore counts sharing a letter are not significantly different ($P > 0.05$). An asterisk (*) indicates significant spore germination ($P < 0.05$). The horizontal dotted line indicates the detection limit (10 CFU mL^{-1}).

López-Pedemonte, Roig-Sagués, Trujillo, Capellas, & Guamis, 2003). Bevilacqua et al. (2007) found a strong antimicrobial activity of lysozyme against *Alicyclobacillus acidoterrestris* spores and vegetative cells in a model system and apple juice, when used in solution in native form or incorporated in water-soluble polyvinyl alcohol (PVOH) film. The antimicrobial activity of lysozyme against vegetative cells could possibly be attributed to the hydrolysis of the β1-4 linkages between *N*-acetylglucosamine and *N*-acetylmuramic acid of the peptidoglycan layer of the cell wall. Cortex hydrolysis by lysozyme along with the release of Ca-DPA and core hydration may positively contribute to the spore germination; however, the antimicrobial action of lysozyme against spores is largely unknown (Bevilacqua et al., 2007; Bevilacqua, Sinigaglia, et al., 2008; Setlow, 2003).

A significant lethality enhancement ($P < 0.05$) at pH 7.0 was also achieved when sodium dodecyl sulfate (SDS), an anionic surfactant, was used at a concentration of 2 g L^{-1} (Fig. 1A). Bactericidal and fungicidal effects of anionic surfactants such as SDS against vegetative cells have been reported by other researchers and attributed to the surfactant-induced cell lysis (Cornett & Shockman, 1978; Vieira & Carmona-Ribeiro, 2006). The mechanisms of action of SDS and other anionic surface-active compounds against bacterial spores are yet to be investigated.

Low-molecular-weight chitosan was effective in increasing spore inactivation at pH ≤ 6.0 (Fig. 1B and C). A synergistic effect was noted for combination of chitosan with SDS, enhancing the PATP lethality by 3.0 logs (Fig. 1D). Different mechanisms for antimicrobial activity of chitosan have been proposed, depending on the pH of the environment and concentration of the protonated (positively-charged) and unprotonated forms of chitosan's amino groups. The bactericidal effect of chitosan appears to be highest at pH levels below 6.0 in which the protonated amino groups ($-NH_3^+$) predominate and chitosan is most soluble. The protonated amino groups may bind to the negatively-charged carboxylate groups ($-COO^-$) located on the surface of the bacterial cell membranes, resulting in weakening and/or disruption of the membranes followed by leakage of cell components (Juneja et al., 2006; Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003). At higher pH levels, the unprotonated amino groups ($-NH_2$) can chelate essential metal ions such as ferric and zinc ions in bacterial cells and, as a result, inhibit the production of toxins and microbial growth (Cuero, Osuji, & Washington, 1991; Juneja et al., 2006; Rabea et al., 2003). Juneja et al. (2006) demonstrated a significant inhibition of *Clostridium perfringens* spore germination and outgrowth by chitosan in ground beef (25% fat) and ground turkey (7% fat) during abusive chilling from 54.4 to 7.2 °C up to a cooling time of 18 h. There is a need to further investigate the lethal effect of chitosan combined with pressure-heat treatment on bacterial spores. Pre-process storage of spore suspension with chitosan at 25 or 45 °C resulted in a significantly lower ($P < 0.05$) spore inactivation by PATP as compared to the pre-process storage at 4 °C (data not shown). It is known that the electrostatic association between the negatively charged SDS micelles and the positively charged chitosan may result in the formation of an insoluble SDS-chitosan complex, if anions and cations are present in the environment at sufficiently high concentrations (Thongngam & McClements, 2005). This might reduce the antimicrobial effectiveness of the compounds. However, the combination of chitosan with SDS was more effective than the individual use of these compounds in enhancing the PATP lethality under our experimental conditions.

Sodium ethylenediaminetetraacetic acid (Na-EDTA) significantly enhanced ($P < 0.05$) the spore inactivation by PATP at pH 5.0 (Fig. 1C). Kalchayanand, Dunne, Sikes, and Ray (2003) reported ≤3.23 log reduction of *Clostridium* spp. spores, including *C. laramie*, *Clostridium sporogenes*, *C. perfringens* and *C. tertium* in roast beef by pressure-heat treatment at 345 MPa and 60 °C for 5 min combined with 500 μg mL^{-1} Na-EDTA and 100 μg mL^{-1} lysozyme, and shelf-life extension of the product up to 7 days at 25 °C.

Fig. 2 shows the inactivation of *B. amyloliquefaciens* spores by a 2-min PATP treatment in the presence or absence of selected commercial natural antimicrobial compounds derived from plant and fruit sources (used at a 2% concentration level) in HEPES buffer (pH 6.8). Extracts of three of these compounds, i.e. licorice, sarsaparilla and yucca, have been used as natural non-ionic surfactants

and antimicrobial compounds in the pharmaceutical and food industries. Shin et al. (2008) and Yang, Kim, Kim, and Oh (2011) previously tested a number of plant and fruit extracts for enhancing thermal inactivation of *B. cereus* spores, and found that grapefruit seed extract increased the spore inactivation at 80 and 85 °C by more than 1.0 log. Grapefruit seed extract has also been reported to have antimicrobial properties against a wide range of Gram-negative and Gram-positive bacteria through disruption of the bacterial membrane as demonstrated by scanning transmission electron microscopy (Heggers et al., 2002).

In the current study, among the 15 different plant and fruit extracts tested, only garlic purée, garlic extract, wasabi, mustard, mustard oil and Japanese apricot (plum) extract significantly enhanced ($P < 0.05$) the PATP lethality against *B. amyloliquefaciens* spores in HEPES buffer (pH 6.8). The level of spore inactivation increased by 1.0–1.5 logs in the presence of these compounds (Fig. 2). Sulfur-containing compounds such as allicin and allyl isothocyanate in garlic, wasabi and mustard have frequently been reported to exhibit inhibitory effects against bacteria and fungi (Ankri & Mirelman, 1999; Shin, Masuda, & Naohide, 2004; Snyder, 1997). Apricot plum extract is known to be rich in phenolic compounds with strong antimicrobial activity against vegetative bacteria including *Salmonella* spp. and *Escherichia coli* O157:H7 (Cevallos-Casals, Byrne, Okie, & Cisneros-Zevallos, 2006). The mechanism of antimicrobial activity of such compounds against bacterial cells may involve a reaction with the cell membrane or inactivation of essential cellular enzymes (Cevallos-Casals et al., 2006; Davidson & Branen, 1981). The mechanisms of action of antimicrobial compounds of plant and fruit sources against bacterial spores need to be studied.

Fig. 3 presents the inactivation of *B. amyloliquefaciens* spores by PATP treatments, up to 8 min, in 0.05 mol L^{-1} HEPES buffer at pH 6.0 with or without addition of chitosan (0.2%), licorice extract (2%) or combination of chitosan and licorice extract (0.2% + 2%) to the spore suspension prior to treatment. The number of germinated spores occurring at various holding times was also determined by calculating the difference in the viable count before and after applying a heat-shock (80 °C, 15 min) to the spore samples. Chitosan was more effective than licorice extract in enhancing the process lethality and a synergistic effect was noted when chitosan was used combined with licorice extract. The viable count reduction during pressure come-up time was also significant ($P < 0.05$) in the spore suspension containing a combination of chitosan and licorice extract. The spore count in the sample containing a combination of chitosan and licorice extract decreased below the detection limit (i.e., ≥7.3 log reduction) after a 5-min PATP treatment. In contrast, the same treatment inactivated the spores by only a 4.6 logs in the buffer with no antimicrobials, and by 5.6 and 4.7 logs in the buffer containing chitosan or licorice extract alone. The highest level of spore germination occurred in the buffer containing both chitosan and licorice extract during PATP treatment for up to 3 min, indicating the contribution of germination to the lethality enhancement of PATP in the presence of antimicrobial compounds.

Licorice extract has previously been reported to contain a number of bioactive flavonoids, including licochalcone A and glabridin with inhibitory effects against vegetative bacteria and fungi (Bassyouni, Kamel, Megahid, & Samir, 2012; Gupta et al., 2008; Fatima et al., 2009; Tsukiyama, Katsura, Tokuriki, & Kobayashi, 2002). No inhibitory or lethal effects of licorice extract on bacterial spores have been reported in the literature to date. None of the other two natural non-ionic surfactants (sarsaparilla and yucca extracts), tested in the initial screening in the current study, were as effective as licorice extract in enhancing the PATP lethality against *B. amyloliquefaciens* spores (data not shown).

3.2. Combined effects of antimicrobial compounds and PATP or TP on spore inactivation in selected low-acid foods

Fig. 4 presents the inactivation of *B. amyloliquefaciens* spores in selected low-acid foods at 600 MPa and 105 °C for 3 min in the presence of 0.2% chitosan or 2% licorice extract and a combination of these compounds (0.2% chitosan + 2% licorice extract). Significant increases ($P < 0.05$) in the spore inactivation by 1.5, 1.4 and 0.9 logs were achieved in carrot purée (pH 5.2), squash purée (pH 5.4) and green bean purée (pH 5.6), respectively by addition of chitosan alone. The addition of licorice extract alone did not significantly enhance ($P > 0.05$) the PATP lethality against *B. amyloliquefaciens* spores in any of the selected low-acid foods tested. Addition of chitosan, combined with licorice extract, was more effective and synergistically enhanced the level of inactivation by PATP in carrot purée, squash purée and green bean purée by 2.2, 2.1 and 1.7 logs, respectively.

Fig. 5 compares the inactivation of *B. amyloliquefaciens* spores by PATP at 600 MPa and 105 °C with that by TP at 105 °C under atmospheric conditions in carrot purée (selected among the three low-acid foods tested) with or without adding antimicrobial compounds. The data shows that the spores were inactivated by PATP at a substantially higher rate than by TP. Furthermore, chitosan with or without licorice extract significantly enhanced ($P < 0.05$) the lethality of both PATP and TP treatments against *B. amyloliquefaciens* spores in carrot purée. The combination of chitosan and licorice extract synergistically enhanced these process lethalities. The spore viable count decreased below the detection limit (i.e., ≥7.2 log reduction) after a 5-min PATP treatment in the presence of combined chitosan and licorice extract, whereas the same treatment resulted in a 5.1-log reduction of spores in carrot purée without added antimicrobials. Addition of chitosan alone (0.2%) or chitosan combined with licorice extract (0.2% + 2%) to the carrot purée resulted in more than 6.0-log reduction in the spore viable count after 60-min of TP, and decreased the spore population to below the detection limit of 10 CFU mL^{-1} after 100 min. In contrast, reductions of only 2.9 and 5.8 logs in the spore viable count after TP treatments at 60 and 120 min, respectively were achieved in the absence of these antimicrobial compounds.

In this study, *B. amyloliquefaciens* TMW 2.479 Fad 82 spore was used as a model non-pathogenic spore. In a recent study, Reddy et al. (2013), reported that *C. botulinum* type A strains and *C. sporogenes*

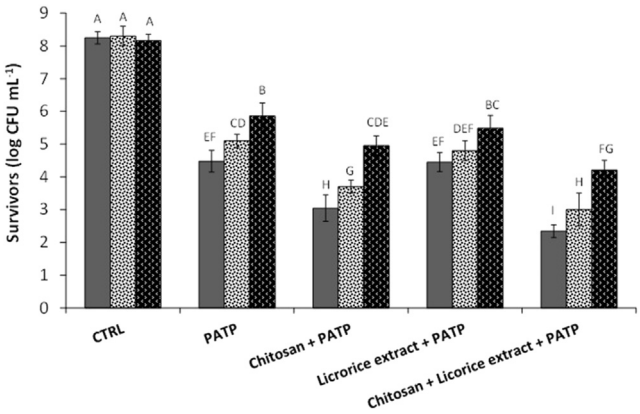

Fig. 4. Inactivation of *Bacillus amyloliquefaciens* spores by pressure-assisted thermal processing (600 MPa and 105 °C, 3 min) in carrot purée (pH 5.2) (▨), squash purée (pH 5.4) (▩), and green bean purée (pH 5.6) (■), without adding antimicrobials, and with addition of chitosan (0.2%), licorice extract (2%), or combination of chitosan and licorice extract (0.2% + 2%). The initial inoculum level was ~1.8 × 10^8 CFU mL^{-1}. Bar graphs and error bars represent the mean survivors (n = 4) and standard deviations, respectively. The means sharing a letter are not significantly different ($P > 0.05$).

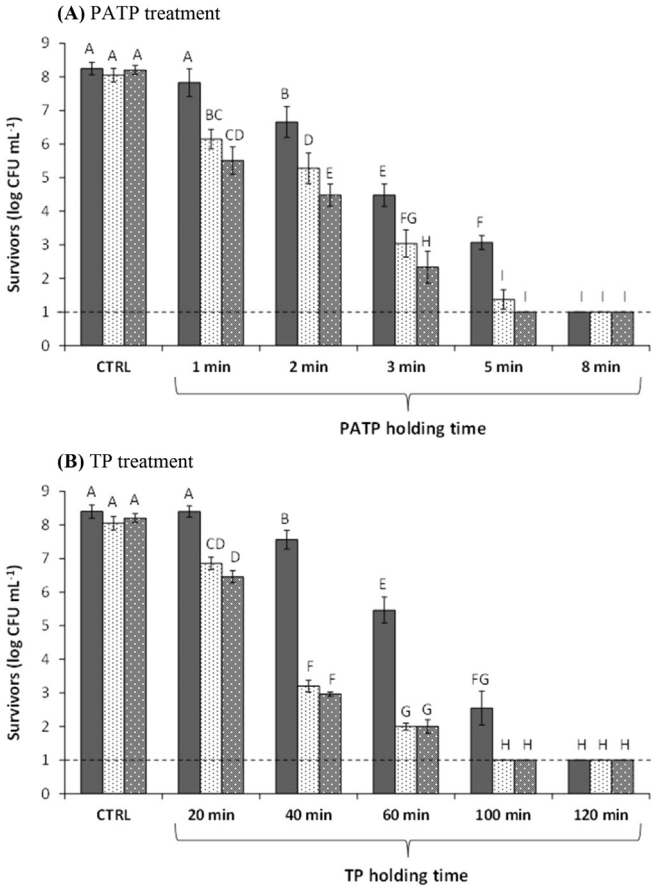

Fig. 5. Inactivation of *Bacillus amyloliquefaciens* spores in carrot purée (pH 5.2) by pressure-assisted thermal processing (PATP) or thermal processing (TP) with or without added antimicrobials. Panel A: PATP at 600 MPa and 105 °C for various holding times; PATP only (■), PATP with addition of 0.2% chitosan (⊡), or PATP in combination of chitosan and licorice extract (0.2% + 2%) (▩). Panel B: Thermal processing at 0.1 MPa, 105 °C for various treatment times and antimicrobial compounds. The initial inoculum level was ~4.5 × 10^8 CFU mL^{-1}. Bar graphs and error bars represent the mean survivors (n = 4) and standard deviations, respectively. The means sharing a letter are not significantly different ($P > 0.05$) (the PATP and TP data were analyzed separately). The horizontal dotted line indicates the detection limit (10 CFU mL^{-1}).

PA3679, tested in *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES) buffer (0.05 mol L^{-1}, pH 7.0) at 105 °C and 700 or 750 MPa, exhibited higher pressure-thermal resistance than that of *B. amyloliquefaciens* TMW 2.479 Fad 82 spores. More studies are necessary to evaluate efficacy of tested antimicrobial compounds on various target pathogenic and surrogate spores of interest.

Although combining PATP or TP with chitosan and licorice extract appeared to be effective in decreasing the treatment time required to inactivate the spores in carrot purée to below the detection limit, the storage stability of the treated product also needs to be investigated. In addition, the potential effects of these compounds on the sensory properties of the product should also be evaluated. For example, Gökmen and Gürbüz (2011) reported that presence of medium-molecular-weight chitosan (0.05%, 0.1% and 0.5%) in Turkish sausage formulation had a positive effect on product's microbiological and sensory quality. However, when used at a larger concentration (such as 1%), it adversely affected the product sensory qualities, including flavor, color, appearance and texture. It is worth pointing out that the concentration of Chitosan used in the present study was lower than that used by Gökmen and Gürbüz (2011). Bento, Stamford, Stamford, de Andrade, and de Souza (2011) reported that the addition of low-molecular-weight chitosan, from *Mucor rouxii*, to bovine meat pâté at 5 mg g^{-1} (0.5%) would be acceptable to consumers, although some negative effects on flavor and taste were reported.

Chitosan obtained through the deacetylation (hydrolysis of acetyl groups) of chitin present within the cell wall of *Aspergillus niger* or the exoskeleton of shrimps is "Generally Recognized as Safe (GRAS)" by the U.S. FDA for use as a food ingredient (U.S. FDA, 2011; U.S. FDA, 2013). Since chitosan is derived from raw materials that are potentially allergenic, concerns about the allergenicity of chitosan may be raised. However, the harsh chemical treatment associated with deacetylation is known to substantially degrade the proteinaceous materials and therefore, the product is not expected to be allergenic (GRAS Associates, 2012). Licorice extract is a GRAS substance and can be used as a safe ingredient in food either as a flavor enhancer or surface-active agent (U.S. FDA, 2012).

4. Conclusions

This study demonstrated that selected antimicrobial compounds could be used to sensitize bacterial spores to subsequent killing by PATP and TP treatments. More specifically, the addition of chitosan alone or chitosan combined with licorice extract (a natural non-ionic surfactant) or SDS (a synthetic anionic surfactant) was most effective in enhancing PATP and TP lethality. Findings of the study could help processors in developing methods to process low-acid foods using less severe heat and pressure conditions than those used currently. Further research is needed to fully understand the mechanisms of antimicrobial action against bacterial spores. This should include investigating the possible interactions between antimicrobials and other macromolecules in a more complex matrix of food systems.

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