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Microbiological efficacy of pressure assisted thermal processing and natural extracts against *Bacillus amyloliquefaciens* spores suspended in deionized water and beef broth

Suresh Devatkal[a,*], *Jeremy Somerville*[a], *Rarinthorn Thammakulkrajang*[a], *V.M. Balasubramaniam*[a,b]

[a] *Department of Food Science and Technology, Parker Food Science and Technology Building, The Ohio State University, 2015 Fyffe Road Columbus, OH 43210, USA*

[b] *Department of Food Agricultural and Biological Engineering, The Ohio State University, 590 Woody Hayes Drive, Columbus, OH 43210, USA*

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ABSTRACT

Effect of aqueous extracts of pomegranate peel (PPE) and tamarind pulp (TPE) on lethality of pressure assisted thermal processing (PATP) against *Bacillus amyloliquefaciens* Fad 82 spores was investigated. *B. amyloliquefaciens* spores (≈10^8 CFU ml^{-1}) were suspended in deionized water (DIW), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer PPE, and TPE (1% w/v in DIW, pH-6) and beef broth with or without PPE and TPE (0.45% w/v). Samples were subjected to pressure assisted thermal processing (PATP, 600 MPa, 105 °C), and thermal processing (TP; 0.1 MPa, 105 °C) for 0 (come-up time), 0.5, 1, 3, and 5 min. Beef broth samples were also pressure treated (HPP; 600 MPa, 35 °C) for 3 and 5 min. Dormant spore survivors were enumerated by spread-plate technique. Heat shock (80 °C for 15 min) was used to enumerate the heat sensitive spores. A 5-min PATP reduced spore survivors counts to below 1-log CFU ml^{-1} (≈6.5–7.0 log reduction) in PPE and TPE as compared to 2.5-log and 3.1-log in DIW and HEPES buffer, respectively. 3 min PATP treatment reduced the spore survival to 3.58 and 2.1 log CFU ml^{-1} in beef broth and beef broth + natural extract, respectively. TP and HPP processing could inactivate the spores only by 0.5–1.2 log CFU ml^{-1}. PPE and TPE are found to enhance the efficacy of PATP.

1. Introduction

Pressure-assisted thermal processing (PATP) is an emerging technology to deliver low-acid shelf-stable foods with improved nutritional and sensory attributes. The treatment involves subjecting pre-heated (75–95 °C) foods to combined high pressure (600–900 MPa) and temperature (90–121 °C) for short holding time. The process has been proposed as a high-temperature short-time process, where both pressure and compression heat contribute to the process's lethality (Balasubramaniam et al., 2008). The major advantage of PATP is the ability to increase product temperature quasi-instantaneously with the heat of compression and subsequently cool the temperature close to its initial value upon depressurization. This reduces the severity of the thermal effect encountered during conventional thermal processing.

* Corresponding author. Present address: National Research Centre on Meat, Chengicherla, Post Box No.19, Boduppal Post, Hyderabad, 500092, India. Tel.: +91 040 29801674x108; fax: +91 040 27201672.
E-mail address: sureshlpt@yahoo.com (S. Devatkal).

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In February 2009, FDA issued a no objection letter to an industry petition for sterilization of a low-acid food product processed by PATP. Although, there are no available commercial food products currently processed by PATP, the technology has potential applications in processing value-added heat-sensitive low-acid shelf-stable products such as soups, egg products, meat products, coffee, tea and mashed potatoes (Balasubramaniam et al., 2008).

Bacterial spores are known to be resistant to high pressure at ambient temperatures (Smelt, 1998). For example, *Bacillus subtilis spores* were shown to survive pressure treatment at 1000 MPa for 40 min at temperature below 10 °C (Nakayama et al., 1996). Further it has been reported that among other *Bacillus* spores, *Bacillus amyloliquefaciens spores* possess very high pressure–heat resistant characteristics under PATP conditions. (Ahn et al., 2007; Olivier et al., 2012). Earlier studies indicated that 5-min pressure only (700 MPa–35 °C) and thermal only (0.1 MPa, 105 °C) treatments inactivated approximately log 0.5 cycle of *B. amyloliquefaciens* spores (Ratphitagsanti et al., 2009). A number of *B. amyloliquefaciens* strains have been proposed as relevant target spoilage organisms for high-pressure thermal processed foods and potential surrogate to assess the efficacy of PATP with respect to proteolytic *Clostridium botulinum* (Olivier et al., 2011).

During PATP treatment, quality deterioration still occurs during pre-heating (75–90 °C). Thus it is desired to evaluate approaches that can reduce severity of pressure–heat treatment during PATP. The synergy of combining antimicrobial compounds with PATP treatment to reduce severity of process and enhance process efficacy has been investigated. A combination of nisin, pediocin and the treatment of 345 MPa, 60 °C for 5 min increased the storage of beef roast inoculated with *clostridial* spores (Kalchayanand et al., 2003). Sucrose laurate at 1% combined with a treatment of 700 MPa, 105 °C showed the synergistic effect on inactivation of *B. amyloliquefaciens* spores suspended in mashed carrot (De Lamo-Castellvi et al., 2010). The addition of organic acids provided significant lethality enhancement against *B. amyloliquefaciens* spores during PATP treatment at 605 MPa, 105 °C for 3 min and further suppressed spore recovery during storage of carrot puree (Ratphitagsanti et al., 2010) crobiology.

Increased consumer demand for natural and minimally processed foods have led to the use of phytochemicals in food preservation. Pomegranate (*Punica granatum* L.) and tamarind (*Tamarindus indica* L.), commonly grown fruits in India are rich sources of natural phytochemicals including tannic acid, tartaric acid, ellagitannins, gallotannins, ellagic acid and gallic acid (Negi and Jayaprakasha, 2003; Fischer et al., 2011). Phenolic compounds extracted from pomegranate peel were characterized using HPLC–DAD–ESI/MS by Fischer et al. (2011) and ellagitannins were found to be the predominant phenolics in all samples investigated. Pomegranate peel ellagitannins, punicalagin, ellagic acid and gallic acid, as natural antimicrobial agents, have been widely exploited against *Staphylococcus aureus* and hemorrhagic *Escherichia coli* for their ability to precipitate membrane proteins and inhibit enzymes such as glycosyl transferases, leading to cell lysis (Braga et al., 2005). Several studies have indicated the potential antibacterial effect of pomegranate peel against different pathogenic bacteria including *S. aureus*, *E. coli*, *Klebsiella pneumonia*, *Proteus vulgaris*, *B. subtilis*, and *Salmonella typhi* (Negi and Jayaprakasha, 2003; Prashanth et al., 2001). Similarly, the antibacterial effect of tamarind against *Bacillus* sp., *S. aureus*, *S. typhi*, *Enterobacter aerogenes*, *E. coli*, *Listeria monocytogenes* and *P. vulgaris* has been demonstrated (Kamble and Patil, 2007; Norhana et al., 2009). Moreover, the use of natural preservatives is desirable to reduce the amount of food additives and provide consumer friendly labeling. Therefore, the application of natural phenolics present in pomegranate peel and tamarind pulp along with pressure assisted thermal processing could provide an opportunity to minimize the process severity and enhance the spore inactivation by PATP.

Therefore, objective of this study was to investigate the feasibility of enhancing the efficacy of PATP against *B. amyloliquefaciens* spores by the application of pomegranate peel and tamarind pulp extracts. A low acid food product (beef broth) was tested to assess the potential food matrix protective effect against spore inactivation under PATP conditions.

2. Materials and methods

2.1. Bacterial strain and preparation of spores

B. amyloliquefaciens TMW 2.479 Fad 82 culture was a kind gift from M. Ganzle (University of Alberta, Edmonton, Alberta, Canada). The culture was grown in trypticase soy broth supplemented with 0.6% yeast extract (TSBYE; Becton & Dickinson U.S.A.) and incubated aerobically at 32 °C for 24 h. After 2 transfers, 100 μL of vegetative cells were spread-plated on nutrient agar supplemented with 0.6% yeast extract (TSAYE; Becton & Dickinson, U.S.A. and 0.001% manganese sulfate ($MnSO_4 \cdot H_2O$, Fisher Scientific, U.S.A.). The inoculated plates were incubated at 32 °C for 3–5 days. Spores were collected by flooding the plates with cold sterile deionized water (DIW) and washed 5 times by differential centrifugation (1500 to 2000 × *g* for 20 min at 4 °C) (Allegra 6R, Beckman Coulter, U.S.A). The spore pellets were resuspended in sterile DIW to obtain approximately 10^8 CFU ml^{-1} spores. The suspension was sonicated for 10 min (SM275HT, Crest, ETL Testing Lab., U.S.A.). Heat treatment (80 °C for 15 min) was applied to destroy any remaining vegetative cells. The spore suspension in deionized water was stored at 4 °C until used.

2.2. Preparation of pomegranate peel (PPE) and Tamarind pulp extracts (TPE)

Pomegranate and tamarind fruits were obtained from a local market. Fruits were washed in running tap water and then rinsed in boiled DIW. Pomegranate was peeled manually and sliced into small pieces. Pulp of tamarind was also separated from peel and seeds. 100 g peel or pulp was blended with 500 ml boiled DIW water in a Warring blender for 1 min at medium speed and allowed for extraction in water bath at 60 °C with intermittent mixing. The extract was filtered in cheese cloth and centrifuged (2000 × *g* for 10 min at 4 °C) and pH was adjusted to 6 using 1 to 5 normal (N) sodium hydroxide The extract was autoclaved (121 °C, 0.1 MPa), and stored at 4 °C. The concentration of solids in these extracts was estimated using a vaccum dryer (1% w/v). Further extract was filtered in 0.45 μm syringe filters (Fisher Scientific, U.S.A) before adding into the spore suspension.

2.3. Preparing spores for processing

Prior to the treatments, the spore suspension was sonicated for 10 min to prevent clumping. Aliquots (0.2 ml) of *B. amyloliquefaciens* spore suspension and 1.8 ml either of sterile 4-(2-hydroxyethyl)-1piperazineethanesulfonic acid (HEPES)

buffer (50 mM, pH-6), DIW, 1% PPE or 1% TPE were aseptically transferred into a sterile pouch (5 × 2.5 cm, polyethylene bags, 01-002-57, Fisher Scientific) to obtain approximately 10^7 CFU ml^{-1}. The pouches were then heat sealed using an impulse heat sealer (American Intl. Electric, U.S.A.) while minimizing occluded air as much as practically possible. All inoculated pouches were stored up to 2 h in an ice-water bath (4 °C) before treatment.

2.4. Pressure-assisted thermal processing

High pressure microbial kinetic tester (PT-1, Avure Technologies Inc., U.S.A.) was used in this study. The unit was equipped with an intensifier (M-340 A, Flow Intl., U.S.A.) that can generate pressures up to 700 MPa. A 54-ml stainless steel pressure chamber was immersed in a temperature-controlled bath. Propylene glycol (Houghton Safe-620TY, Houghton Intl. Inc., U.S.A.) was used as the heat transfer medium in the temperature-controlled bath. The glycol was also used as the pressure-transmitting medium within the high pressure processor. For PATP applications, the temperature of the external glycol bath was set at 105 °C (target process temperature) to minimize any loss during the test. The high pressure processor had a pressurization rate of 14.3 MPa s^{-1}, while depressurization occurred within 2 s for all treatments. The sample temperature, bath temperature, and chamber pressure were recorded every second with a *K*-type thermocouple sensor (model KMQSS-04OU-7, Omega Engineering, U.S.A.) and pressure transducer (model 3399 093 006, Tecsis, Germany) using a data acquisition computer. For various combined pressure–heat treatment experiments, the pouch was placed inside a sample holder, which was made of a 10-ml polypropylene syringe (model 309604, Becton & Dickinson, U.S.A.) wrapped with two layers of insulating material (Sports Tape, CVSR Pharmacy Inc., U.S.A.). The void volume in the syringe was filled with approximately 8 ml of water to ensure that the spore suspension and the syringe water in the vicinity of the sample experience similar thermal response during processing (Rajan et al., 2006). Insulating syringe also helped minimize the heat exchange between the syringe containing the spore sample and the surrounding glycol bath, which has higher heat of compression than that of water. The syringe containing the pouch was preheated at an empirically determined (Nguyen et al., 2007) preprocessing temperature (approximately ≈54.6 °C [Table 1]) in a water bath (Isotemp 928, Fisher Scientific) for 2 min. The syringe was then immediately loaded into the pressure chamber. The pressure–temperature histories in the vicinity of the sample within the syringe were recorded. After reaching the predetermined temperature, T2 (Table 1), the pressurization process started. Test samples were treated for PATP (600 MPa–105 °C for 0/come-up time 0.5,1, 3, and 5 min). The pressure holding time did not include the pressure come-up time or the depressurization time. After depressurization, the spore suspension was immediately removed from the pressure chamber and cooled in an ice-water bath (4 °C) to prevent further inactivation. Surviving *B. amyloliquefaciens* populations were enumerated immediately. All experiments were independently repeated at least 3 times.

2.5. Thermal processing

Thermal processing (TP) experiments were carried out at 105 °C and 0.1 MPa using a 35-L circulating oil bath (NESLAB EX-35 Digital One, Thermo Fisher Scientific Inc., U.S.A.). Thermal process experiments utilized custom-fabricated thermal death-time disks (TDT disk; 18 mm diameter, 4.5 mm height) as sample holders (Jin et al., 2008). The sample temperature was monitored and recorded by inserting a *K*-type thermocouple (Omega Engineering) attached to a data logger (IOtech Inc., U.S.A.) into a TDT disk containing sterile DIW without spores. One water bath and two oil baths were used to manipulate thermal preprocessing time to that of PATP (Nguyen et al., 2007). First, the sample disks were pre-heated in a water bath at 54.6 °C and then transferred to the first oil bath that was maintained at 113 ± 1 °C. Once the sample temperature reached approximately 100 °C, all TDT disks were shifted to the second oil bath, which was maintained at 105.5 °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 inactivation. Temperature histories were automatically recorded by the data logger. Come-up time of the samples to reach 105 °C was approximately 2.20 min and it took approximately 2 min to bring the thermal-processing temperature from 105 °C to 4 °C Surviving *B. amyloliquefaciens* populations were enumerated within 3 h after the treatments. All experiments were independently repeated at least 3 times.

2.6. High pressure processing, PATP and TP of beef broth inoculated with Bacillus amyloliquefaciens spores and natural extracts

The combined effect of PATP and natural extracts was tested using inoculated beef broth (Meijer, U.S.A.). Through a separate set of preliminary experiments, it was found that untreated beef broth supported the growth of the

Table 1 – Pressure-thermal histories for various hold times during (A) pressure assisted thermal processing (600 MPa and 105 °C).

Holding time (min)	Come-up time (min)	Pre-process (T1, °C)	Just before pressurization (T2, °C)	Immediately after pressurization (T3, °C)	Pressure holding (T3–T4 °C)[a]	After de-pressurization (T5, C)[b]
0	0.65 ± 0.01[c]	55.03 ± 0.03	65.96 ± 0.04	106.68 ± 0.44	–	82.91 ± 0.23
0.5	0.63 ± 0.02	55.23 ± 0.07	64.40 ± 0.17	105.93 ± 1.22	107.0 ± 0.67	81.45 ± 0.30
1	0.64 ± 0.02	55.30 ± 0.12	68.23 ± 0.40	104.84 ± 0.95	106.30 ± 1.09	82.53 ± 0.40
2	0.65 ± 0.03	55.03 ± 0.12	68.81 ± 0.14	104.90 ± 1.08	105.20 ± 1.18	81.91 ± 0.30
3	0.65 ± 0.02	54.93 ± 0.12	67.81 ± 0.14	105.10 ± 1.08	105.30 ± 1.18	79.91 ± 0.30
5	0.65 ± 0.02	55.07 ± 0.10	68.74 ± 0.28	105.35 ± 1.1	104.22 ± 1.19	76.89 ± 0.50

[a] External glycol bath surrounding the pressure chamber was maintained at 105 °C.
[b] Depressurization occurred within 2 s.
[c] Data presented are means ± standard error of three independent trials.

B. amyloliquefaciens spores. Spores inoculated into beef broth at low levels (approximately log 2 CFU ml^{-1}) were able to grow to >log 6 CFU ml^{-1} after 2 d of storage at 32 °C. Sterile beef broth had an initial pH value of 5.5. The pH of the beef broth was adjusted to pH 6 using 1 N to 5 N sodium hydroxide. Aliquots of natural PPE and TPE (0.9 ml) were added to the sterile beef broth (0.9 ml), and thoroughly mixed to obtain a final concentration of natural extract to 0.45% w/v. Beef broth-natural extracts mixture (1.8 ml) and *B. amyloliquefaciens* spores (0.2 ml) were mixed and packaged as described earlier. Various beef broth samples inoculated with *B. amyloliquefaciens* spores were immediately processed at 600 MPa–35 °C (HPP), 600 MPa–105 °C (PATP) and 0.1 MPa–105 °C (TP) for 3 and 5 min. Surviving *B. amyloliquefaciens* populations in treated beef broth pouches were enumerated immediately after the treatments. All experiments were independently repeated at least 3 times.

2.7. Enumeration of spore survivors

Pouches and thermal death time disks containing spores were aseptically opened and used for estimating spore count. A tenfold serial dilutions of the treated spore suspension were prepared in sterilized 0.1% peptone water (Difco, BD). The aliquots of the appropriate dilutions were then spread-plated on duplicate TSAYE. Colonies of survivors were counted after incubation at 32 °C for 48 h. Inactivation of *B. amyloliquefaciens* by HPP, PATP and TP was calculated as the difference between the logarithmic counts of colonies in untreated and treated samples.

2.8. Enumeration of heat sensitive spores

PATP treated pouches containing spores suspended in beef broth were aseptically opened and divided into two equal parts. One part was used for estimating spore count as described above. The remaining portion of the treated spore sample was heat shocked at 80 °C for 15 min to identify heat sensitive spores after PATP treatments. Serial dilutions and enumeration by spread-plate technique were performed as described earlier. Colonies of survivors were counted after incubation at 32 °C for 48 h (Wuytack et al., 2000). The percentage of heat sensitive spore was estimated from the knowledge of the total surviving population after PATP (N_{PATP}) and the number of dormant spores survived PATP treatment followed by a heat shock ($N_{PATP+HT}$) at 80 °C for 15 min. Population of heat sensitive spore was calculated using the formula:

$$\%\ \text{heat sensitive spore} = \frac{(N_{PATP} - N_{PATP+HT})}{N_{PATP}} \times 100.$$

2.9. Statistical analysis

Data analysis was performed using the Statistical Analysis System software (SAS 9.1, SAS Inst. Inc., U.S.A.). The independent variables were suspension media and various pressure-holding times. The spore survivors (log CFU ml^{-1}) in response to the combined pressure–heat process treatment served as the dependent variable and the experimental replications were also included as a blocking factor in the analysis. Mean comparison was evaluated with the Student–Newman, Keuls (SNK) test at a 5% significant level ($P = 0.05$).

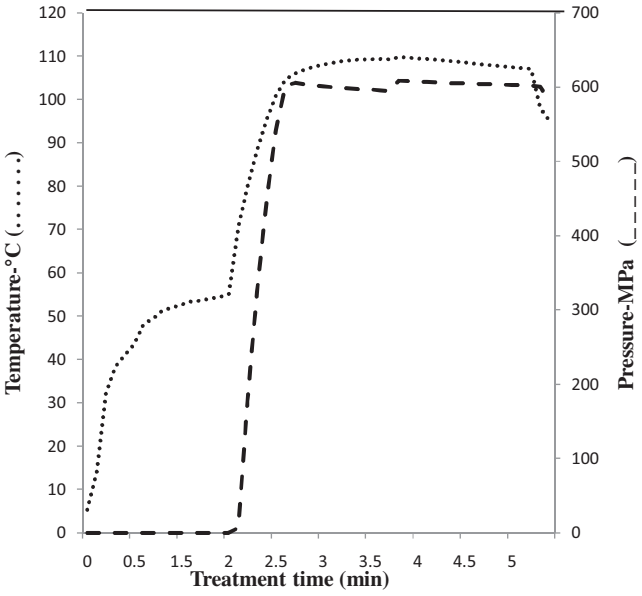

Fig. 1 – Representative pressure–temperature history during a 3-min pressure assisted thermal processing.

3. Results and discussion

3.1. Temperature and pressure histories during PATP and TP

Table 1 presents summary of pressure–temperature history during PATP of *B. amyloliquefaciens* spores suspended in different medium. For PATP, pouches were pre-heated in water bath (54.6 °C) for 2 min. During subsequent loading into the pressure vessel, pouches initially lost some temperature and then after loading, pouches gained heat from glycol bath maintained at 105 °C. The average temperature just before pressurization was 67.32 °C. After pressurization (average come-up time was 38.55 s) pouches gained the temperature very rapidly and average temperature reached 105.46 °C. Average process temperature during the pressure (600 ± 5 MPa) holding time was 105.73 °C (Fig. 1). After completion of hold time, pouches were depressurized (depressurization time 2 s) and the temperature decreased to 80.9 °C. These pouches were immediately transferred to ice-water bath for further microbial analysis.

During TP also, thermal histories of the samples were matched similar to that of PATP. Average pre-heating temperature was 54.9 °C, and samples were held at an average temperature of 105.16 °C. After completion of different holding time, samples were immediately cooled in ice-water bath to an average temperature of 3.23 within 2.10 min.

3.2. Influence of natural extracts in enhancing spore inactivation during pressure-assisted thermal processing and thermal processing

Preliminary experiments verified that addition of natural extracts to the spore suspension (without TP or PATP treatment) did not cause any statistically significant inactivation. The comparison of the spore survivors after PATP treatments in different suspension media is shown in Fig. 2. Although bacterial spores are inactivated as a function of pressure, process temperature and hold time, different spores have different resistances during the pressure come-up time and

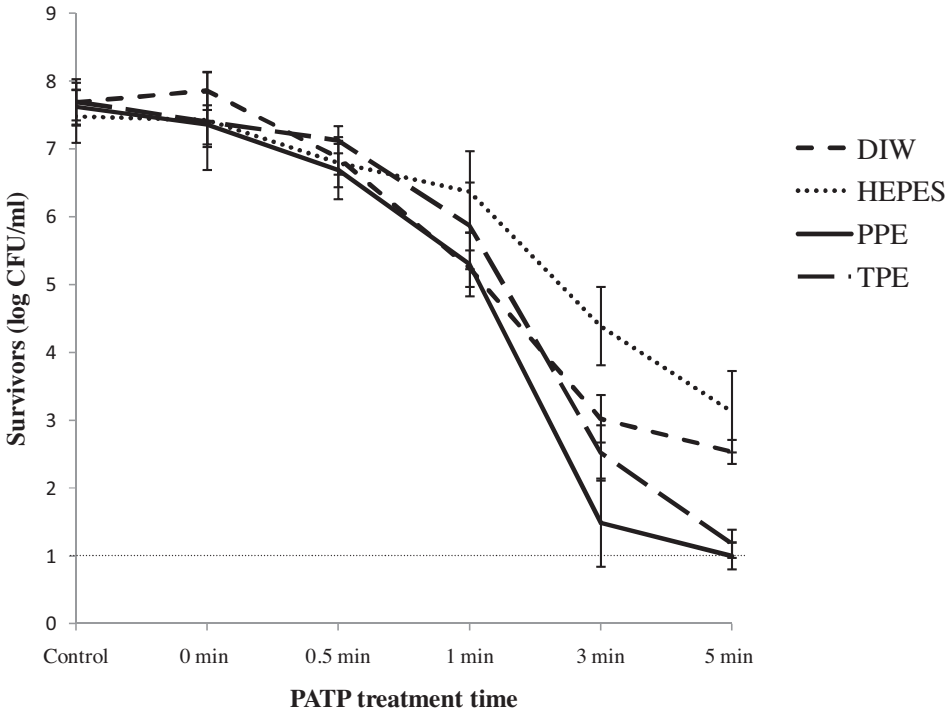

Fig. 2 – Surviving population of *Bacillus amyloliquefaciens* spores suspended in DIW, HEPES buffer PPE and TPE after combined pressure (600 MPa) and heat (105 °C) treatment up to 5 min treatment time. The treatment time did not include pressurization (38 s) and depressurization (2 s) time.

considering this come up time inactivation is important in determining the overall effect of PATP. During PATP come-up time (approximately 38.5 s), the reduction in number of spores was about 0.3 log cycles in spores suspended in 1% PPE and TPE and no reduction in spore survivors was observed for spores suspended in DIW and HEPES buffer. Similarly, Margosch et al. (2004) also reported a reduction in the population (0.5-log reduction) of *B. amyloliquefaciens* spores during a 5 min pressure come-up time for PATP at 600 MPa and 80 °C. They further reported a reduction in numbers of *C. botulinum* TMW 2.357 (1.5-log reduction), *C. thermosaccharolyticum* (3-log reduction), and *B. subtilis* (0.5-log reduction) spores during similar come-up times. Ratphitagsanti et al. (2010) also observed a 0.4-log reduction during PATP come-up time. However, Rajan et al. (2006) reported that a 42 s come-up time during PATP (0.1–700 MPa and 95, 105, 110 and 121 °C, 0–15 min) resulted in no or limited log reduction of *B. amyloliquefaciens Fad 82* spores. In the present study, shorter holding time (600 MPa at 105 °C for 0.5 to 1 min) resulted in limited reduction in the spore counts. PATP holding time of 1 min resulted in 2.45, 1.1, 2.32 and 1.83 log reductions in DIW, HEPES, PPE, and TPE suspended spores, respectively. A treatment of 3 min PATP holding time resulted in 4.69, 3.09, 5.13 and 5.18 log reduction in DIW, HEPES, PPE, and TPE suspended spores, respectively. Thus increasing PATP pressure holding time increased *B. amyloliquefaciens* spores inactivation. Further, *B. amyloliquefaciens* spores were ultimately inactivated to below the detection level ($\leq$10 CFU ml^{-1}) after 5 min-PATP holding time in PPE. In TPE, the number of spore survived after 5 min holding time were slightly higher (1.18-log) than the detection limit of 1.0-log. But the same 5 min PATP holding time was ineffective to inactivate spores to below the detection level when suspended in DIW and HEPES buffer. Therefore, natural extracts enhanced PATP efficacy ($P > 0.05$) and provided higher inactivation as compared to spores suspended in DIW and HEPES buffer. Comparative log reductions in PATP treated spores have been reported in earlier studies. Margosch et al. (2006) have reported more than 4-log reductions in *B. amyloliquefaciens* Fad 82 spores suspended in Tris–His buffer and treated at 1200 MPa, 120 °C for 2 min. Rajan et al. (2006) observed a 5-log reductions in *B. amyloliquefaciens* spores suspended in egg patty mince and treated at 700 MPa, 105 °C for 3 min. Similarly Ahn et al. (2007) reported a 5-log reductions in spores of *B. amyloliquefaciens* suspended in DIW and treated at 700 MPa, 105 °C for 2 min. Ratphitagsanti et al. (2010) reported that PATP (700 MPa, 105 °C) pressure holding time of 1 and 2 min resulted in 4 and 5.8-log reduction of *B. amyloliquefaciens* spores, respectively, and after 3 min-PATP holding time spores were ultimately inactivated to below the detection level. De Lamo-Castellvi et al. (2010) observed that spores suspended in 0.5 and 1.0% sucrose laurate solutions were reduced to the below detection level (10 CFU ml^{-1}) after a 2-min treatment at 700 MPa and 105 °C.

Thermal treatment (105 °C and 0.1 MPa, 5 min) did not result in significant ($P < 0.05$) reduction of spores during come-up time and up to 2 min (Fig. 3). However, 5 min holding time resulted in inactivation of spores by 0.56, 1.61 and 1.22-log in HEPES, PPE and TPE extract, respectively. Ratphitagsanti et al. (2010) reported that combining the organic acids (100 mM) with pressure (700 MPa and 35 °C) or thermal (105 °C and 0.1 MPa) treatments did not enhance *B. amyloliquefaciens* spore inactivation and resulted only in 0.1 to 0.4-log reduction. Similarly, De Lamo-Castellvi et al. (2010) found that sucrose laurate combined with TP or HPP (come-uptime, 1, 2, and 5 min) showed no inactivation, but a slight increase in spore viability.

Antimicrobial effect of pomegranate peel and tamarind extracts has been reported in earlier studies (Negi and Jayaprakasha, 2003). However, the sporicidal efficacy of natural phenolics during thermal and PATP processing is not documented. Endospores have multiple, distinct layers that contribute to resistance and metabolic dormancy (Black et al.,

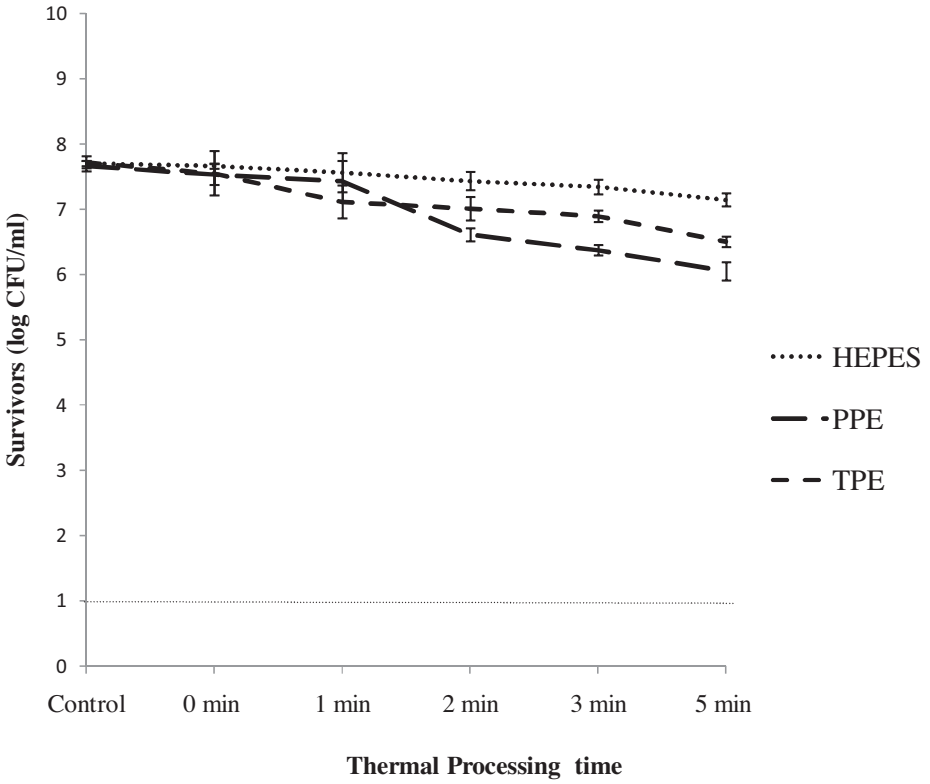

Fig. 3 – Surviving population of *Bacillus amyloliquefaciens* spores suspended in DIW, HEPES buffer PPE and TPE after heat (105 °C) treatment over 5 min treatment time.

2008). Dehydration of spore core contributes to endospore resistance. Although the mechanism of spore killing is unclear, disruption of inner membrane of endospores during PATP might rehydrate the core and allows inactivation of endospores by antimicrobials (Melly et al., 2002). A treatment of clostridial endospores at 600 MPa and 90 °C for 8 min reduced viable spore counts by more than 3 log cycle and resulted in the release of more than 95% DPA but did not affect the membrane fluidity (Hofstetter et al., 2013). Also, antimicrobial substances (nisin and reutericyclin) had a divergent effect on endospore membrane structure during thermal and pressure assisted thermal processing. It was concluded that pressure mediated spore inactivation and release of DPA do not require disturbance of highly ordered state of endospore membranes.

3.3. *Influence of natural extracts in enhancing spore inactivation during pressure-assisted thermal processing of B. amyloliquefaciens suspended in beef broth*

Beef broth supported the growth of *B. amyloliquefaciens* spores. Further it was also noticed that natural extracts added to beef broth and spore suspension did not cause any inactivation in the absence of PATP, HPP and TP. Figs. 4(a) and 3(b) shows a comparison of survivor of *B. amyloliquefaciens* spores suspended in sterile beef broth with or without natural extracts and treated with PATP (600 MPa, 105 C), TP (0.1 MPa, 105 °C) and HPP (600 MPa, 105 °C) for 3 and 5 min, respectively. In general, PATP inactivation of *B. amyloliquefaciens* spores was enhanced by use of 0.45% PPE and TPE. The inactivation in PATP-3 min holding time was 4.18, 5.57 and 5.56-log CFU ml^{-1} in beef broth, beef broth + PPE and beef broth + TPE, respectively. Further increasing the PATP holding time to 5 min inactivated the spores to below detection limit (<10 CFU ml^{-1}) in beef broth + PPE and beef broth + TPE suspended spores. However, PATP holding time 5 min inactivated only 5-log CFU ml^{-1} in spores suspended in beef broth without natural extracts. When we compare the food matrix data with DIW and HEPES buffer for an equivalent PATP time, different trends were observed. For PATP-3 min, spore survivors in beef broth were higher than DIW but lower than HEPES buffer. For PATP-5 min, slightly higher spore survivors were observed in beef broth than DIW but lower than HEPES buffer. During PATP-3 min holding time, the spores suspended in beef broth + PPE, survivors were higher in beef broth than PPE suspended spores. In contrast, the survivors of spores suspended in beef broth + TPE were lower than in TPE. During PATP-5 min holding time no difference was observed between spores suspended in beef broth + natural extracts only natural extracts as PATP with 5 min reduced the spore survivors to below detection limits (<log 10 CFU ml^{-1}). Results of thermal processing showed that TP with 3 min holding time inactivated the spore by 0.1, 0.5 and 0.7 log in beef broth, beef broth + PPE and beef broth + TPE, respectively. Further increasing the TP holding time to 5 min did not cause any significant reduction in the survivors of spores suspended in beef broth + PPE and beef broth + TPE. Further, HPP did not result in any significant inactivation of *B. amyloliquefaciens* spores. These results indicated that inactivation of *B. amyloliquefaciens* spores in PATP was significantly ($P < 0.05$) higher than thermal and high pressure processing at an equivalent process temperature and pressure, respectively. (Fig. 4a and b). Previous studies have also demonstrated the resistance of *B. amyloliquefaciens* spores to high pressure at ambient temperature and thermal treatment at atmospheric pressure. De Lamo-Castellvi et al. (2010) observed that *B. amyloliquefaciens* spores subjected to TP (0.1 MPa,105 °C and HPP (700 MPa, 35 °C) did not show any inactivation and the use of sucrose laurate also did not enhance the spore

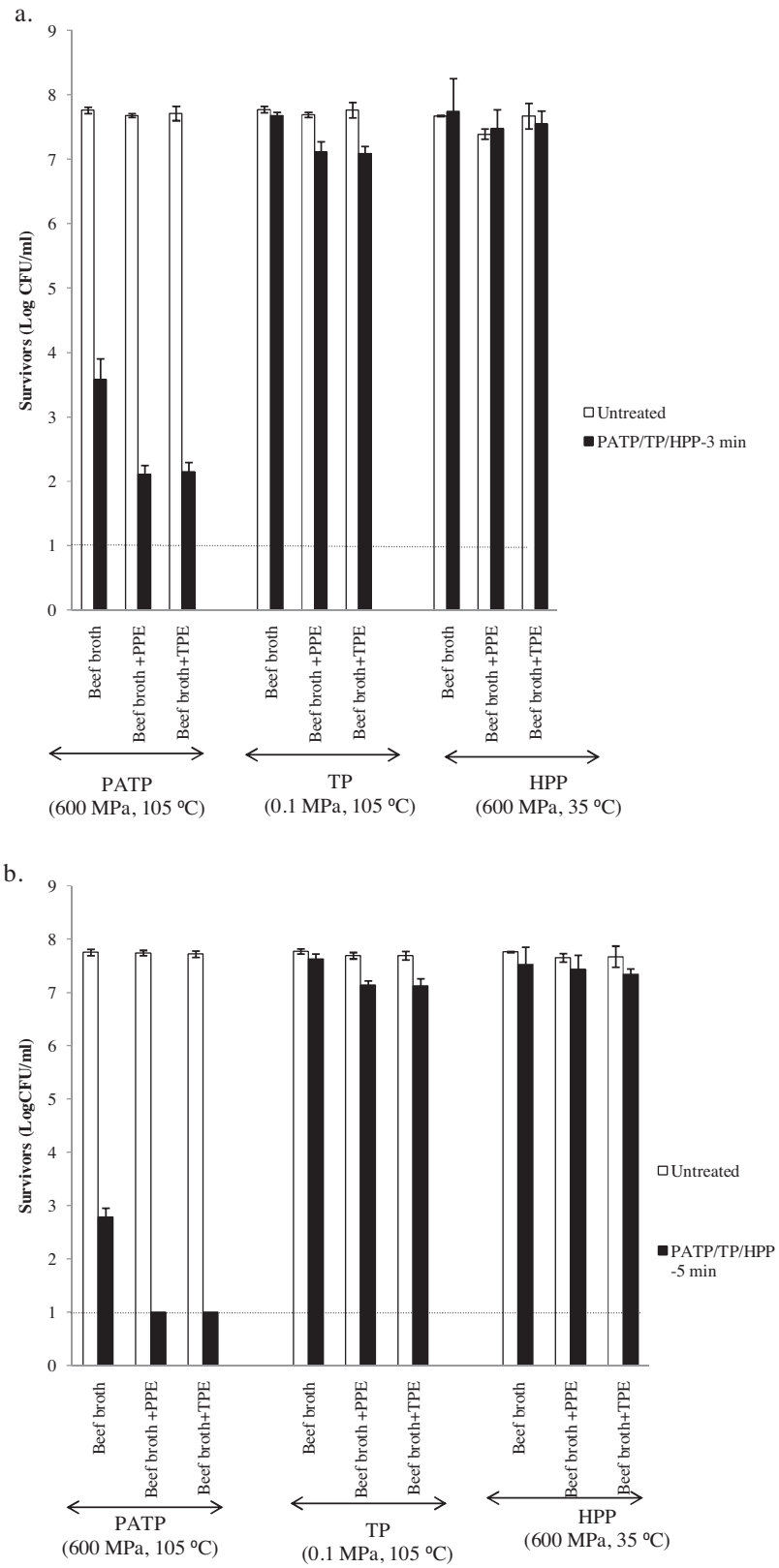

Fig. 4 – Surviving population of *Bacillus amyloliquefaciens* spores suspended in sterile beef broth with or without 0.45% PPE and TPE after PATP (600 MPa, 105 °C and TP (0.1 MPa, 105 °C) and HPP (600 MPa, 35 °C) treatments for 3 min (a) and 5 min (b).

inactivation by TP and HPP in mashed carrot. Similarly, Ratphitagsanti et al. (2010) reported that combining organic acids with HPP (700 MPa, 35 °C) and TP (0.1 MPa, 105 °C) resulted in only 0.3 log reduction of *B. amyloliquefaciens* spores.

Earlier studies on protective effect of food matrix during PATP indicated that milk with 0, 2, and 4% fat concentration did not provide any protective effect on *B. cereus* spores during a treatment of 600 MPa, 40 °C for 2 min (Raso et al., 1998). Similarly, egg patties with complex ingredients did not provide a protective effect on the inactivation of *G. sterothermophilus* spores when treated at 700 MPa and 105 °C (Rajan et al., 2006). But, Ananta et al. (2001) observed that *B. sterothermophilus* spores were protected by surrounding food matrix. Similarly, De Lamo-Castellvi et al. (2010) observed the protective effect

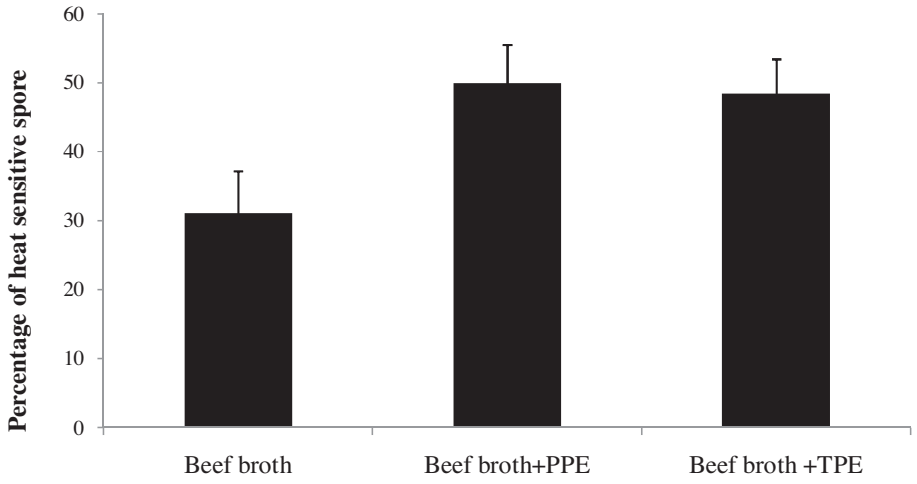

Fig. 5 – Percent heat sensitive surviving *Bacillus amyloliquefaciens* spore suspended in beef broth in the presence of PPE and TPE after 600 MPa–105 °C for 3 min.

of mashed carrot on inactivation of *B. amyloliquefaciens* spores during a treatment of 700 MPa, 105 °C for 2 min. These differences in the results could be attributed to the non-uniform water activity and differences in adiabatic heating of the product, pressure medium and the heat transfer from the vessel wall (Georget et al., 2014). In general, the food system itself could have a protective effect on the spores because certain ingredients such as fats, sugars, and salts can interact with bacterial spores in a protective way and lower the water activity of the food that could lead to retarded inactivation (Sevenich et al., 2013).

3.4. *Heat sensitivity of PATP/HPP treated B. amyloliquefaciens spores suspended in beef broth and natural extract*

It has been well established that spore inactivation under PATP conditions proceeds as a two step process. First, spores are induced by high pressure to rapidly release Ca–DPA, and the partly hydrated spores are subsequently killed by the combination of pressure and heat due to their increased overall stress sensitivity (Margosch et al., 2004; Reineke et al., 2011). Most of existing mechanistic studies have also reported that the release of dipicolinic acid is accompanied by a loss of heat resistance of endospores during PATP (Black et al., 2008; Paidhungat et al., 2002). In our study, PATP with 3 min treatment resulted in a significant ($P < 0.05$) heat sensitive spore population among the survivors of *B. amyloliquefaciens* spores suspended in beef broth (Fig. 5). Further addition of natural extracts also increased the number of heat sensitive spores among PATP survivors. In contrast, a treatment of PATP-5 min did not result in a significant heat sensitive spore population among the surviving *B. amyloliquefaciens* spores. Reineke et al. (2013) reported that the loss of heat resistance caused by rapid hydration of the spore core can be strongly accelerated by increasing the treatment temperature (>60 °C) and pressure (>500 MPa). Hence, the number of inactivated spores is same as the number of heat sensitive spores during PATP with 5 min treatment. Thus the combination of high pressure and high temperature directly affects the inner spore membrane and/or membrane channel proteins, leading to a spore core hydration and a subsequent inactivation, presumably due to a full loss of barrier properties of the inner spore membrane (Reineke et al., 2013).

4. Conclusions

Natural extracts (pomegranate peel and tamarind pulp) enhanced the efficacy of PATP (600 MPa, 105 °C up to 5 min) against *B. amyloliquefaciens* spores suspended in DIW and beef broth. PATP with 3 min resulted in significant number of heat sensitive spores and addition of natural extracts also resulted in increased number of heat sensitive spores during PATP with 3 min holding time. Use of natural extracts as food additives may help in addressing the consumer demands for products with highly acceptable sensory, nutritive qualities, and safety. Understanding the stability and antimicrobial efficacy of natural antimicrobial compounds after combined pressure–heat treatment are worth further investigation.

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