# High pressure processing pretreatment enhanced the thermosonication inactivation of Alicyclobacillus acidoterrestris spores in orange juice

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**Submission date:** 19-Feb-2019 07:02PM (UTC+0700)
**Submission ID:** 1080204314
**File name:** Evelyn_2016b.pdf (679.15K)
**Word count:** 7195
**Character count:** 37845

Food Control 62 (2016) 365–372

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

# High pressure processing pretreatment enhanced the thermosonication inactivation of *Alicyclobacillus acidoterrestris* spores in orange juice

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ARTICLE INFO

*Article history:*
Received 29 June 2015
Received in revised form
29 October 2015
Accepted 4 November 2015
Available online 10 November 2015

*Keywords:*
Ultrasound
Heat
Thermal
Pasteurization
Modelling
Predictive microbiology

ABSTRACT

The spoilage of high acid fruit juices and nectars by *Alicyclobacillus acidoterrestris* is a major concern to juice manufacturers around the world since it is difficult to detect. In this study, thermosonication (ultrasound and heat, TS) and thermal inactivation of *A. acidoterrestris* spores in pretreated orange juice were carried out and resistance parameters were estimated. First, the effect of TS acoustic energy density (AED, 0.3–20.2 W/mL) on the inactivation at 75 °C was investigated. Then, the influence of TS temperature (70–78 °C) on the spore inactivation (AED = 20.2 W/mL) was studied. Next, we explored the effect of high pressure processing (HPP) pretreatment of juice on the 20.2 W/mL TS inactivation at the best temperature (78 °C). Lastly, the thermal inactivation of spores in juice heat shocked + 1 min sonicated vs. untreated juice was also investigated.

Results of TS showed higher spore inactivation for higher AED ($D_{75°C}$-value of 49 min for 20.2 W/mL vs. 217 min for 0.33 W/mL). Lower *D*-values were obtained at higher temperatures ($D_{78°C}$-value of 28 min vs. $D_{70°C}$-value of 139 min at 20.2 W/mL). The TS $D_{78°C}$-value (at 20.2 W/mL) decreased further from 28 min to 14 min when the orange juice was previously submitted to 600 MPa for 15 min. Thermal treatment alone at 78 °C resulted in almost no spore inactivation, whereas the heat shock + ultrasound pretreatment of juice enhanced the thermal inactivation of spores ($D_{85°C}$-value decreased from 69 to 29 min). To conclude, HPP-assisted TS provided the best method for spore inactivation, indicating the benefit of high pressure and power ultrasound technology in addition to heat. TS required at least 8 °C lower temperatures than thermal treatments to achieve the same spore inactivation, which could enhance juice quality and energy savings.



## 1. Introduction

*Alicyclobacillus acidoterrestris* (AAT) is an aerobic, rod-shaped, gram-positive, endospore-forming bacterium which is able to grow at a pH range of 2.0–7.0 and a temperature range of 25–60 °C. Optimal growth occurs at a pH of around 4.0–4.5, and a temperature around 40–45 °C (Bevilacqua, Sinigaglia, & Corbo, 2008a). The spores of AAT survive the thermal pasteurization (generally between 80 and 100 °C) employed by the fruit beverage industry, and exhibit very high heat resistance compared with major spoilage microbes of high-acid shelf-stable foods (1.0 min < $D_{95°C}$ < 5.3 min and 6.0 min < $D_{90°C}$ < 23.0 min) (Silva & Gibbs, 2001; Silva, Gibbs, Nunez, Almonacid, & Simpson, 2014). AAT spore germination and growth up to a level of $10^5$–$10^6$ cfu/mL can occur after pasteurization (cycle of up to 5 days) in high-acid fruit juices when the storage and distribution temperatures are around 40 °C (Splittstoesser, Churey, & Lee, 1994). Product spoilage is difficult to detect visually since AAT does not produce gas during growth. However, juice/beverage spoilage is evident by the off flavour, caused by guaiacol and other halophenols (ppb) (Gocmen, Elston, Williams, Parish, & Rouseff, 2005). Therefore, AAT was suggested as reference microorganism for pasteurization processes in high-acid fruit products (Silva & Gibbs, 2001; 2004).

Large-scale AAT spore germination and spoilage was first reported in 1982 in aseptically packaged apple juice (Cerny, Hennlich, & Poralla, 1984). Since then, other incidents have been reported in

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http://dx.doi.org/10.1016/j.foodcont.2015.11.007
0956-7135/

USA, Europe and Japan (Jensen, 2000) and in different types of fruit products such as lemonade carbonated fruit juice drinks, shelf-stable ice tea containing berry juice, fruit pulps, and canned diced tomatoes (Duong & Jensen, 2000; Pettipher & Osmundson, 2000; Walls & Chuyate, 1998). Today, food and beverage spoilage by AAT spores has become an industrial issue.

The effectiveness of heat alone or combined with antimicrobials for inactivating AAT has been investigated: nisin was added to fruit juices (Komitopoulou, Boziaris, Davies, Delves-Broughton, & Adams, 1999), chlorine dioxide was added to the surface of apples (Lee, Gray, Dougherty, & Kang, 2004), grape polyphenols were added to grape juice (Oita & Kohyama, 2002), enterocin AS-48 was added to fruit juices (Grande et al., 2005), ascorbic acid was added to apple juice (Bahçeci & Acar, 2007), and eugenol and cinnamaldehyde were added to acidified malt extract broth (Bevilacqua, Corbo, & Sinigaglia, 2008b).

A number of different non-thermal technologies and their combination with heat have also been investigated for microbial spore inactivation in juices, fruit products and other foods (Evelyn, Kim, & Silva, 2016). These include high hydrostatic pressure combined with heat or HPP-thermal (Evelyn & Silva, 2015a; S. Lee, Chung, & Kang, 2002; Shearer, Hoover, Dunne, & Sikes, 2000; Silva, Tan, & Farid, 2012; Sokołowska et al., 2012), high pressure carbon dioxide (Bae, Lee, Kim, & Rhee, 2009; Casas, Valverde, Marín-Iniesta, & Calvo, 2012), and radiation (Nakauma, Saito, Katayama, Tada, & Todoriki, 2004). Power ultrasound is another non-thermal method that has been studied for microbial spore (Evelyn & Silva, 2015b, 2015c) and enzyme inactivation (Sulaiman, Soo, Farid, & Silva, 2015). Ultrasonic waves at sufficient intensity can cause microbial cell death by a phenomenon called cavitation (Chen, 2012). The microgas bubbles are formed during the rarefaction cycle of the acoustic wave within a liquid, collapse violently during the compression cycle (Leong, Ashokkumar, & Kentish, 2011), and create micro-mechanical shocks leading to disruption of cellular components and hence cell lysis (Guerrero, López-Malo, & Alzamora, 2001). Lower decimal reduction values (*D*-values) of bacterial and mould spores were registered after simultaneous use of ultrasound and heat (thermosonication [TS]) and ultrasound-assisted (before or after) thermal processing (Burgos, Ordonez, & Sala, 1972; Evelyn & Silva, 2015b, 2015d; Garcia, Burgos, Sanz, & Ordonez, 1989; López-Malo, Palou, Jiménez-Fernández, Alzamora, & Guerrero, 2005; Ordonez & Burgos, 1976).

To date, limited information is available on the inactivation of AAT by power ultrasound, especially on spores (Ferrario, Alzamora, & Guerrero, 2015; Wang, Hu, & Wang, 2010; Yuan, Hu, Yue, Chen, & Lo, 2009). Therefore, in this research, orange juice inoculated with AAT spores was processed by TS. The effects of varying energy density, temperature, and juice pretreatments were investigated, and the spore first-order TS resistance parameters (*D*- and z-values) were determined and compared with thermal inactivation processes. The specific objectives were: (i) to determine the best acoustic energy density (AED) for TS inactivation at 75 °C; (ii) to determine the effect of TS temperature on the *D*-values, (iii) to study the effect of high pressure pretreatment on the TS spore inactivation and compared with thermal inactivation alone; (iv) to compare the thermal resistance of spores in orange juice pretreated with ultrasound vs. no pretreatment; and (v) to recommend optimal TS conditions for the pasteurization of orange juice.

## 2. Material and methods

### 2.1. *A. acidoterrestris* microbiology

#### 2.1.1. Strain

*Alicyclobacillus acidoterrestris* type strain NZRM 4447 (same as ATCC 49025 and NCIMB 13137) was obtained from the New Zealand Reference Culture Collection. This strain was isolated from apple juice concentrate. It was precultured on potato dextrose agar (PDA, Difco North Ryde, Australia) adjusted to a pH 4.0 with 10% w/v (0.1 g/mL) tartaric acid. The PDA plates were incubated at 45 °C for 3 days and used as source of inoculum for sporulation.

#### 2.1.2. Sporulation

The sporulation procedure described by Silva et al. (2012) was used. Briefly, the fresh cells from the initial culture were inoculated on PDA (pH 5.6) and incubated for 21 days at 45 °C to obtain spores. The spores were confirmed by phase contrast microscopy (Motic microscope BA410 Series, Canada). Then the spores were harvested by flooding the plates with 1–2 mL of sterile distilled water and dislodging the spores from the agar surface with a sterile bent glass rod. After harvesting, the spores were washed three times by centrifugation with sterile distilled water (Centrifuge Sigma 4K15, UK) at 4,000 g and 4 °C for 10 min, resuspended in 50 mL sterile phosphate buffer (pH 7.2), and stored at 2 °C until use.

#### 2.1.3. Orange juice inoculation

The orange juice used in this study (pH 3.8, 9.5 ± 0.1°Brix) was purchased from a local supermarket and used as the treatment medium for the AAT spore inactivation. The juice contained added pulp, flavour, food acid (citric acid), and preservatives (potassium sorbate). For 0.33 W/mL TS experiments, a small portion (ca. 1–2 mL) of spore solution was inoculated into 99 mL of orange juice, whereas for thermal and other TS experiments, 1 mL of the spore solution was inoculated into 49 mL of orange juice. A final spore concentration of approximately $10^6$ or $10^7$ cfu/mL was obtained in orange juice.

#### 2.1.4. Spore enumeration

The *A. acidoterrestris* spore concentration in the juice before and after processing was determined by spread plating into acidified (pH 4) PDA plates. The spore concentration before processing was determined after a heat shock treatment (80 °C, 10 min) of 5 mL juice in a thermostatic water bath to eliminate any vegetative cells. Orange juice samples were decimal diluted ten times with 0.1% (w/v) sterile buffered peptone water (Difco, Becton Dickinson, USA). Each tube dilution was mixed repeatedly using a high-speed vortex mixer to yield a uniform spore suspension, and plated twice. The PDA plates were then inverted inside a sealed plastic bag, to avoid drying of the medium and keep the moisture away from the agar surface, and incubated at 45 °C for 3–5 days. Plates showing 30 to 300 colonies were used for enumeration, and spore concentration was expressed in colony forming units per milliliter (cfu/mL) of juice sample after calculations for corresponding dilution.

### 2.2. Experimental design and data analysis

#### 2.2.1. Experimental design

The first experiment examined the effect of TS acoustic energy density (AED) at 75 °C on AAT spore inactivation in orange juice. The AED of 0.33, 4.10 and 20.20 W/mL were used and evaluated, with 20.20 W/mL being the maximum energy of the equipment for the tip and juice volume used in the TS process. Because AED 20.20 W/mL was also the best performing AED, the following TS experiments were carried out at 20.20 W/mL. In the next experiment, TS inactivation of AAT spores was carried out at three temperatures (70, 75, and 78 °C) for up to 60 min; 78 °C is the maximum temperature recommended for this ultrasound unit by the manufacturer. Thirdly, we investigated the effect of TS on AAT spore inactivation with and without juice HPP pretreatments, and compared with TS and thermal inactivation alone at 78 °C, the best

temperature. Two 15 min HPP pretreatments at 200 and 600 MP were attempted in order to improve the TS inactivation of *A. acidoterrestris* spores.

Lastly, thermal inactivation of AAT spores in orange juice was carried out at three temperatures (78, 85, and 95 °C) for orange juice pretreated with a heat shock followed by 1-min ultrasound (16.20 W/mL, see Section 2.5.1) and untreated orange juice. The results were also compared with the TS results.

#### 2.2.2. Data analysis

TableCurve 2D version 5.01 (SYSTAT Software Inc., USA) was used to fit the 1st order kinetics to the linear spore survival lines, and estimate the kinetic parameters ($D_T$- and $z$-values). $D_T$-value is the time required at a certain temperature to reduce the microbial population by 90%, whereas $z_T$-value (°C) is the temperature change that results in a 10-fold change in the $D_T$-value. Mean square error (MSE) and coefficient of determination ($R^2$) were used to evaluate the goodness of the fit. For each temperature/method at least two survival experiments were carried out and the $D$-values were estimated by linear regression of logarithmic number of survivors ($\log N/N_0$) versus time. Then, the average $D_T$-value ± standard deviation (SD) was calculated for each temperature/treatment. A $t$-test with significance assigned at $P < 0.05$ was used to compare any two $D$-values or any two log reductions for different processing conditions/methods (Statistica 8, Statsoft Inc., USA).

### 2.3. Power ultrasound unit

An UP200S ultrasonic processor by Hielscher (Hielscher-Ultrasonic Gmbh, Germany) was used for the induction of ultrasonic waves. The processor has a high frequency (24 kHz) and was operated at 100% amplitude and continuous energy supply. Sonotrodes with a tip-diameter of 3 mm (460 W/cm$^2$, 33 W) and 14 mm (105 W/cm$^2$, 162 W) were used to generate different AEDs (Hielscher, 2007). The sonotrode coupled to the ultrasonic processor via the horn, amplified the vibrations and transferred them to the orange juice to be sonicated. The maximum temperature supported by the sonotrode was 78 °C.

### 2.4. Thermosonication inactivation of *A. acidoterrestris* spores in orange juice

#### 2.4.1. HPP pretreatment of orange juice

A high-pressure food processing system (QFP 2L-700, Avure Technologies, Columbus, Ohio, USA) was used for pressure treatment of AAT spores. The maximum pressure handled by the distilled water (the working fluid) was 690 MPa. Pressure come-up times were ≤1.5 min and depressurization took less than 30 s. Plastic pouches (16 × 16 cm, Cas-Pak, New Zealand) containing the inoculated orange juice (50 mL) were vacuum packed and thermosealed (Multivac C200, Germany). Then, the juices were submitted to 200 or 600 MPa HPP for 15 min at a temperature below 39 °C. The treated juices were subsequently submitted to thermosonication as explained in the following section.

#### 2.4.2. Thermosonication of orange juice

Three different AED levels were used: 0.33, 4.10 and 20.20 W/mL. For 0.33 W/mL, the TS experiment was carried out using the procedure described in Evelyn and Silva (2015b) with the 3 mm tip-diameter sonotrode. The ultrasonic treatments were carried out inside the biosafety cabinet (ESCOAC2-E/S, Singapore) to prevent contamination of the sample. Briefly, 100 mL of orange juice was added to a round-bottomed flask which was placed inside a thermostatic water bath for better control of the temperature during the process. After preliminary trials to obtain the working temperature for the TS treatment, the juice was preheated to an initial temperature. Next, aseptic inoculation was carried out by adding 1–2 mL of inoculum and ultrasound was switched on for the sonication treatment. Treatment times included the time to reach 75 °C (≤2 min of sonication). This temperature was maintained constant (±1 °C) throughout the experiments. Juice samples (0.5 mL) were taken from the flask at specific processing times after inoculation and cooled in an ice water bath for spore survivor counts.

With respect to 4.10 and 20.20 W/mL TS, the Hielscher's stainless steel D14K temperature-controlled flow vessel was filled with 8 mL orange juice containing the spores. A probe with a 3 mm diameter tip was used to obtain 4.10 W/mL, whereas a 14 mm diameter probe was used to generate the AED of 20.20 W/mL. The vessel was also initially preheated up to an initial temperature (also determined from preliminary trials), filled with unprocessed juice, and then the ultrasonication treatment time count started. The times to reach the desired temperatures were quick (≤1 min), and these were included in the TS treatment times. The juice was maintained at the desired temperature (±0.5 °C), which was recorded by a thermocouple (connected to a computer) at the vessel outlet. This temperature was kept constant by continuously running cooling water from the tap through the jacket of the vessel. The juice was treated for specific processing times, cooled in an ice water bath and spore survivor counts were immediately performed. The procedure was repeated for other specific processing times.

### 2.5. Thermal inactivation of *A. acidoterrestris* spores in orange juice

#### 2.5.1. Pretreatment of orange juice

Heat shock (HS, 80 °C for 10 min) followed by 1-min ultrasonication of juice containing the spores was attempted to improve thermal inactivation, since past results showed HS + ultrasonication of *Clostridium perfringens* spores enhanced its thermal inactivation (Evelyn & Silva, 2015c). Briefly, for the heat shock, a test tube containing 10 mL of juice with the *A. acidoterrestris* spores (~ $10^7$ cfu/mL) was thermally processed at 80 °C for 10 min in a water bath and then placed in an ice water bath and submitted to 1-min ultrasound by submerging the 14 mm tip ultrasound sonotrode in the juice. The resulting AED was 16.20 W/mL.

#### 2.5.2. Thermal processing of orange juice

Thermal processing of orange juice was carried out for treated (HS followed by 1-min ultrasound) and untreated orange juice. Transparent food grade sterile pouches (8 × 8 cm, Cas-Pak, New Zealand) were filled with 3 mL of treated or untreated juices, and thermally processed at 78, 85, and 95 °C for various times in a water bath. The thermally treated samples were taken out from the water bath at different time intervals and kept in an ice water bath until microbial enumeration.

## 3. Results and discussion

### 3.1. Effect of TS acoustic energy density on *A. acidoterrestris* spore inactivation in orange juice at 75 °C

The effect of TS acoustic energy densities (0.33, 4.10 and 20.20 W/mL) on the AAT spore reduction in orange juice at 75 °C for 60 min is illustrated in Fig. 1. The first order kinetics of the TS inactivation was supported by the low MSE value (0.0001–0.004) and high $R^2$ (0.850–0.997). Our results showed higher spore inactivation for higher AED ($D$-values decreased with the increase in AED): a $D_{75°C}$-value of 49 min for 20.20 W/mL compared to 97 min for 4.10 W/mL and 217 min for 0.33 W/mL. The $z$-value for the effect of AED on $D$-values was 36 W/mL ($R^2$ = 0.85). López-Malo et al. (2005) also found lower $D$-values for mould spores at the highest

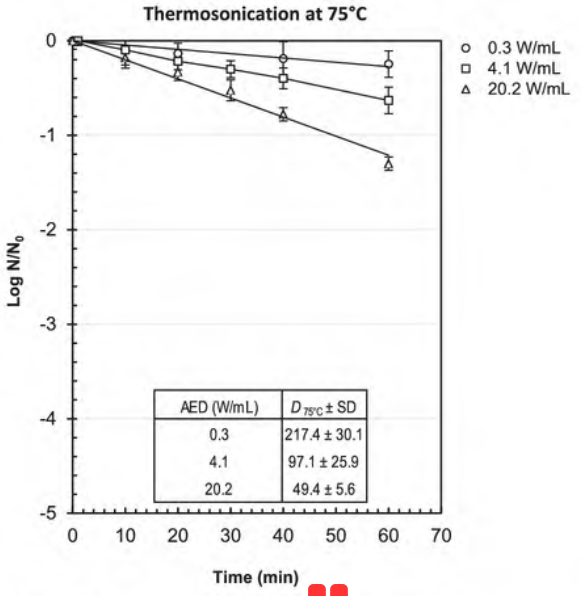



**Fig. 1.** Effect of acoustic energy density (AED) on the thermosonication inactivation of *A. acidoterrestris* spores in orange juice at 75 °C.

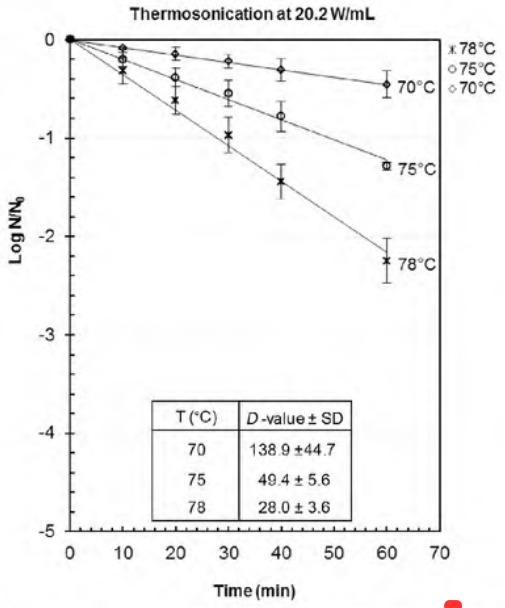



**Fig. 2.** Effect of temperature on the thermosonication (20.2 W/mL) inactivation of *A. acidoterrestris* spores in orange juice.

amplitude of 120 μm at 20 kHz: $D_{60°C}$-value of 0.8 min for *Aspergillus flavus* and $D_{52.5°C}$-value of 3.8 min for *Penicillium digitatum* in Sabouraud broth. Previous investigators have also observed that greater log reductions in AAT vegetative cells occur during TS (23–25 kHz) when the energy is increased from 200 to 600 W at 25–50 °C (Wang et al., 2010; Yuan et al., 2009). Linear, biphasic linear and Weibull models have been used to predict vegetative AAT cells (DSM 3922 and DSM 14558) in apple juice (Wang et al., 2010; Yuan et al., 2009).

Except for the aforementioned data on vegetative cells, there have been no reports on TS inactivation of AAT spores or other bacterial spores for different AED. Since the highest spore inactivation was obtained with 20.20 W/mL, the TS inactivation of AAT at 20.20 W/mL at different temperatures was further investigated and results are discussed in the following section.

### 3.2. Effect of temperature on the TS inactivation of *A. acidoterrestris* spores in orange juice

The log survivors of AAT spores under 20.20 W/mL TS processing in orange juice at three temperatures (70, 75 and 78 °C) are shown in Fig. 2. As expected, TS temperature plays a significant role in spore inactivation, with greater spore inactivation at higher temperatures. The 20.20 W/mL TS *D*-values obtained were 28 min at 78 °C, 49 min at 75 °C and 139 min at 70 °C, with *z*-value of 11.5 °C (Table 1). The first-order kinetic model showed again good performance indices (0.0001–0.013 MSE, 0.959–0.998 $R^2$), and also good fit for the effect of temperature on *D*-values ($R^2$ = 0.99), confirming the linearity of the TS death curves. The higher spore inactivation at the higher temperature for the survival curves are in agreement with our past results with *Bacillus cereus* spores in skim milk (Evelyn & Silva, 2015b) and the result of Garcia et al. (1989) with *Bacillus subtilis* spores in whole milk and glycerol.

### 3.3. Effect of high pressure pretreatment on *A. acidoterrestris* spore inactivation at 78 °C

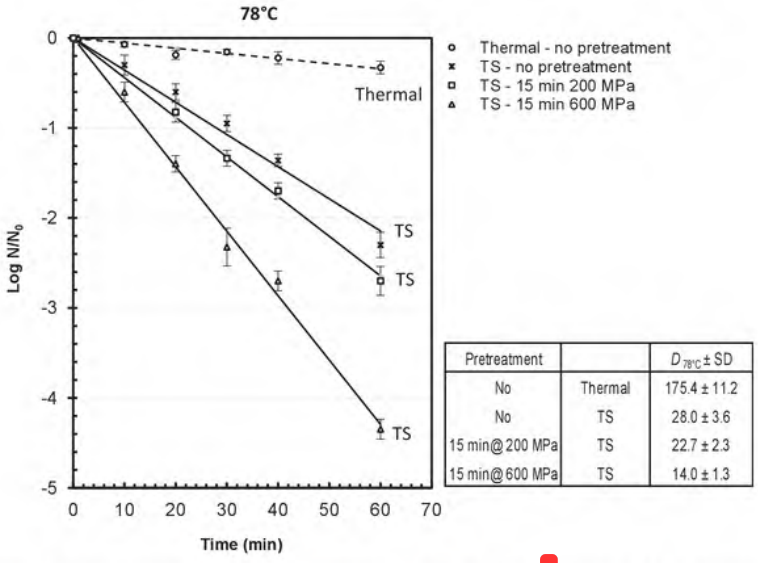
Fig. 3 shows the log survivors of AAT spores at 78 °C up to 60 min with HPP juice and its comparison with thermosonication (untreated) and thermal alone. For untreated juice at 78 °C (the maximum temperature supported by the ultrasound probe), AAT log reductions by TS (2.3 log) was far higher than after the thermal process (0.3 log) of 60 min ($P < 0.05$). The $D_{78°C}$-values were 14–28 min for TS and 175 min for thermal processing, indicating the remarkable advantage of ultrasound technology. The higher spore inactivation by TS than by thermal processing at the same temperature is in agreement with previous experiments carried out with other spore species (Evelyn & Silva, 2015b; Garcia et al., 1989; López-Malo et al., 2005; Wordon, Mortimer, & McMaster, 2012).

Both HPP pretreatments enhanced the AAT spore inactivation in orange juice by TS. HPP at 600 MPa showed a better performance (4.4 log after 60 min, $D_{78°C}$-value = 14 min) than 200 MPa HPP (2.7

**Table 1**
$D_T$- and *z*-values for thermosonication (20.2 W/mL) and thermal inactivation of *Alicyclobacillus acidoterrestris* spores in orange juice[a].

| $D_T$-value ± SD (min) at: | No pretreatment of juice | | Heat shock (80 °C, 10 min) followed by 1-min ultrasound pretreatment of juice |
|---|---|---|---|
| | Thermosonication | Thermal | Thermal |
| 95 °C | nd | 1.5 ± 0.2 | 0.8 ± 0.1 |
| 85 °C | nd | 69.4 ± 2.3 | 29.3 ± 1.2 |
| 78 °C | 28.0 ± 3.6 | 175.4 ± 11.2 | 103.1 ± 8.1 |
| 75 °C | 49.4 ± 5.6 | nd | nd |
| 70 °C | 138.9 ± 44.7 | nd | nd |
| *z*-value ± SE (°C) | 11.5 ± 0.3<br>$R^2$ = 0.99 | 8.0 ± 0.3<br>$R^2$ = 0.95 | 7.9 ± 0.3<br>$R^2$ = 0.97 |

nd = not determined.
SD = standard deviation.
SE = standard error.
[a] *A. acidoterrestris* strain NZRM 4447 (ATCC 49025, NCIMB 13137) was used; *D*-values are means ± standard deviation and obtained from two survival experiments; The first order kinetic parameters for both processes showed good performance indices (0.0001–0.120 MSE and 0.910–0.998 $R^2$).




| Pretreatment | | $D_{78°C}$ ± SD |
|---|---|---|
| No | Thermal | 175.4 ± 11.2 |
| No | TS | 28.0 ± 3.6 |
| 15 min@200 MPa | TS | 22.7 ± 2.3 |
| 15 min@600 MPa | TS | 14.0 ± 1.3 |

**Fig. 3.** Effect of high pressure processing pretreatments on the thermosonication (20.2 W/mL, 78 °C) inactivation of *A. acidoterrestris* spores in orange juice.

log after 60 min, $D_{78°C}$-value = 23 min) ($P < 0.05$), followed by TS without pretreatment, which produced inactivation that was similar to using 200 MPa pretreatment (2.3 log after 60 min, $D_{78°C}$-value = 28 min). It is known that pressures between 100 and 800 MPa can initiate the germination of spores (Gould & Sale, 1970; Paidhungat & Setlow, 2002; Wuytack, Boven, & Michiels, 1998), thus making the spores more susceptible to subsequent inactivation treatments (Black et al., 2007; Sarker, Akhtar, Torres, & Paredes-Sabja, 2015). However, long treatment times (>10 min) were still needed to significantly reduce the number of AAT spores in juice.

### 3.4. Thermal inactivation of *A. acidoterrestris* spores in orange juice pretreated with ultrasound vs. no pretreatment

Fig. 4 compares the log AAT survivors for juice submitted to HS followed by 1-min ultrasound vs untreated juice at temperatures of 78, 85, and 95 °C. HS followed by 1-min ultrasonication of AAT spores in the juice decreased ($P < 0.05$) the thermal resistance of AAT spores approximately by half at 85 and 78 °C. For example, the $D_{85°C}$-value was reduced from 69.4 to 29.3 min, and the $D_{78°C}$-value decreased from 175.4 to 103.1 min (Table 1), confirming the benefit of HS + ultrasound to enhance the thermal inactivation. At 95 °C the difference was less marked. Damage of AAT spores might occur after HS + ultrasound thus further sensitizing the spores to subsequent heat treatment. We have previously shown that HS + 1 min ultrasound pretreatment before thermal processing doubles the rate of *C. perfringens* spore inactivation in beef slurry (Evelyn & Silva, 2015c) vs untreated spores: $D_{105°C}$-value of 1.1 min vs. 2.5 min, $D_{100°C}$-value of 3.4 min vs. 7.1 min, and $D_{95°C}$-value of 9.8 min vs. 21.7 min. The mechanisms for the inactivation are still unknown and need to be explored. Thermal inactivation experiments of juices pretreated with 1-min ultrasound but not heat shocked were also carried out. However, a negligible effect was observed on the spore thermal susceptibility, with $D_{95°C}$-value = 1.3 min similar to 1.5 min of untreated spores, and *z*-value of 8.0 °C similar to 7.8 °C obtained with untreated spores. Previous investigators found no change on the thermal resistance of *Clostridium* spp. spores after exposure to 15–36 W/mL ultrasound treatments alone (Broda, 2007; Evelyn & Silva, 2015c; Goodenough & Solberg, 1972). However, other investigators have reported a decrease in the spore thermal resistance of several species of *Bacillus* after the 12–15 W/mL ultrasound treatments (Burgos et al., 1972; Ordonez & Burgos, 1976), indicating that different species respond differently to the inactivation process.

The thermal log survivors for pretreated and untreated juice were also linear with 0.0004–0.120 MSE, 0.910–0.996 $R^2$, and the *D*-values temperature dependence ($R^2 \geq 0.95$) (Fig. 4, Table 1). The $D_{85°C}$-value of 69 min for untreated orange juice was similar to the value obtained (66 min) by Silva, Gibbs, Vieira, and Silva (1999) with the same strain in orange juice. $D_{85°C}$-values between 50 and 94.5 min were determined with spores from AAT strains 46, 70, 145 and DSM 2498 in orange juice (Eiroa, Junqueira, & Schmidt, 1999). The $D_{95°C}$-value of 1.5 min was also in the range of previous reported values (1.0–5.3 min) (Baumgart, Husemann, & Schimdt, 1997; Eiroa et al., 1999; Komitopoulou et al., 1999; Splittstoesser et al., 1994; Walls, 1997). The estimated *z*-values of 7.9–8.0 °C were comparable to the values found (7.7–7.8 °C) by other investigators (Pontius, Rushing, & Foegeding, 1998; Silva et al., 1999; Splittstoesser et al., 1994).

### 3.5. TS vs thermal inactivation of *A. acidoterrestris* spores in orange juice

The first-order kinetic parameters for TS and thermal treatment in orange juice are shown in Table 1. TS was a better process than thermal processing for AAT spore inactivation, producing a 6-fold reduction in the *D*-value at the same temperature: $D_{78°C}$-value of 28 min for TS vs 175 min for thermal ($P < 0.05$). Similar *D*-values were obtained for TS at 8 °C lower temperatures than for thermal processes ($P > 0.05$). For example, the TS $D_{70°C}$-value was not significantly different from thermal $D_{78°C}$-value. Past reports have

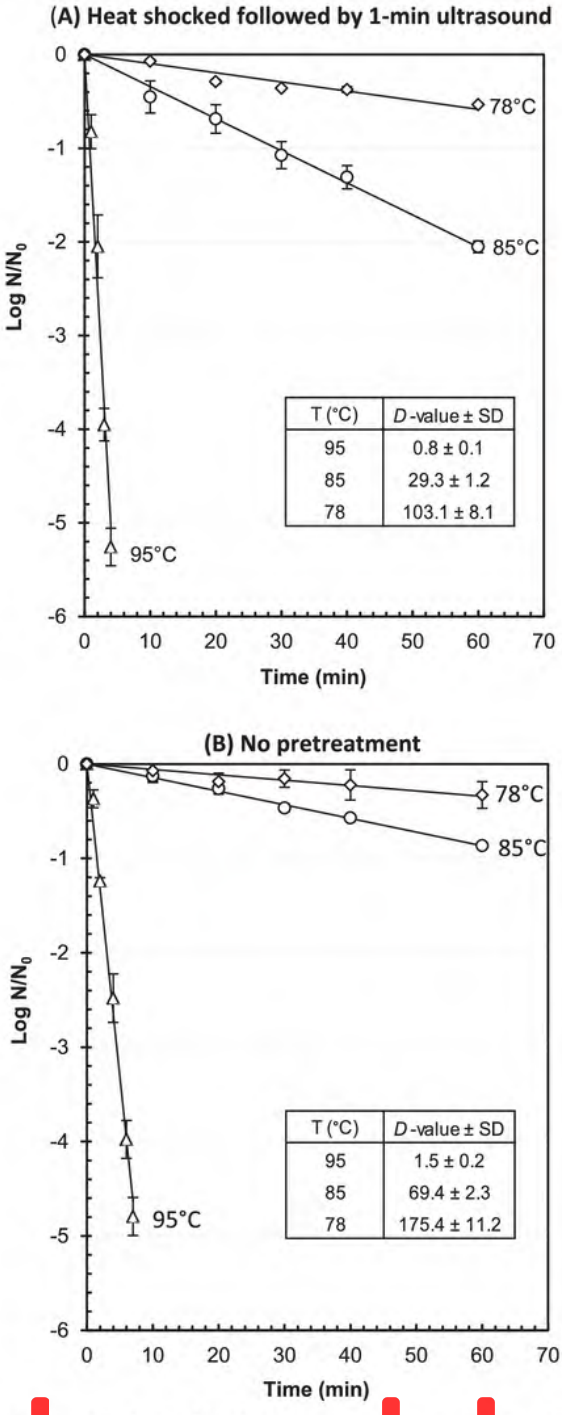



**Fig. 4.** Thermal inactivation of *A. acidoterrestris* spores in orange juice (A - heat shock followed by 1-min ultrasound pretreatment of juice; B - no pretreatment of juice).

shown the benefit of ultrasound technology on thermal spore and vegetative cell inactivation (lower temperatures and processing times to achieve the same lethality values), suggesting less negative impact of heat in the food quality (Evelyn & Silva, 2015b, 2015d; Garcia et al., 1989; López-Malo et al., 2005; Wordon et al., 2012).

The lower thermal *z*-value (8.0 °C) than TS *z*-value (11.5 °C) obtained for AAT spores in this study (Table 1) means the spores are more susceptible to temperature changes in thermal processes than TS processes. The result is in agreement to our previous result using *B. cereus* spores in milk (Evelyn & Silva, 2015b). Garcia et al. (1989) also reported an increase in the TS *z*-values (9.1–9.4 °C in milk, 13.4–14.4 °C in glycerol), which is thought to be related to the changes in the properties of medium used during heating and ultrasound processes (Evelyn & Silva, 2015b; López-Malo et al., 2005; Mason, Paniwnyk, & Lorimer, 1996).

### *3.6. Recommendations of TS and thermal pasteurization conditions for orange juice*

The typical level of microbial contamination of fruit juice is around $10^2$–$10^3$ cfu/mL (Ho, Doona, Kustin, & Feeherry, 2010). Therefore, and to ensure total microbial inactivation, a 5 or 6D pasteurization is often recommended for fruit juices (Gaze & Betts, 1992; Sant'Ana, Alvarenga, & Pena, 2014; USFDA, 1998). However, given the extreme thermal resistance of AAT spores compared with other fruit spoilage organisms, Silva and Gibbs (2001; 2004) recommended a minimum pasteurization of 2 to 3D. High temperature short time (HTST) pasteurization at 90–95 °C for 15–30 s are normally used to pasteurize orange juice (Braddock, 1999). These thermal pasteurization conditions are not sufficient to inactivate AAT spores ($D_{95°C}$ = 1.5 min), since only ≥4.5 min at 95 °C will deliver 3D (3 log reductions in AAT spores). With respect to TS, 78 °C was the maximum temperature allowed by the ultrasound unit. However, the $D_T$-value of the orange juice TS pasteurization can be estimated for higher temperatures based on the TS *z*-value = 11.5 °C (Table 1): 6.89 min at 85 °C, 2.53 min at 90 °C, 0.93 min at 95 °C, 0.34 min at 100 °C and 0.13 min at 104 °C. Consequently, a TS of at least 2.8 min at 95 °C or 23 s at 105 °C are recommended for orange juice pasteurization by TS (20.2 W/mL), which will ensure the minimum 3 log reduction on the *A. acidoterrestris* spores in orange juice.

## 4. Conclusion

TS of orange juice pretreated with 600 MPa HPP for 15 min was the best technique to inactivate *A. acidoterrestris* spores, allowing a 3 log reduction after 42 min. TS AED and temperature are important determinants of AAT spore inactivation, with greater inactivation occurring at higher AED/temperature. The pretreatment of juice with a heat shock (80 °C, 10 min) followed by ultrasound, duplicated the spore thermal inactivation. However, overall TS was 6-fold more effective than thermal treatments in reducing AAT spores in orange juice and required an 8 °C lower temperature to obtain the same inactivation rates as the conventional thermal process. The thermosonication and thermal spore inactivation followed first order kinetics. The results demonstrated the advantage of high pressure-assisted thermosonication for the inactivation of *A. acidoterrestris* spores in orange juice. However, further studies are needed to investigate the impact of the long processing time on the juice sensory/quality attributes and to design ultrasound probes that can withstand higher temperatures, thus allowing lower processing times for the same spore inactivation.

## Acknowledgement

The PhD grant no. 246/D4.4/PK from the Directorate General of Higher Education, Ministry of Education and Culture of Indonesia is acknowledged. Project no. 3701175 "Non-thermal pasteurization of foods", funded by the Faculty of Engineering Research

Development Fund, University of Auckland is also acknowledged. Particular thanks to my colleague Rafael for supplying the *A. acidoterrestris* microbial culture. The support from laboratory and administrative staff from the Chemical and Materials Engineering Department, University of Auckland, is also appreciated.

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