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PII: S1466-8564(18)30669-6
DOI: https://doi.org/10.1016/j.ifset.2019.01.007
Reference: INNFOO 2114

To appear in: *Innovative Food Science and Emerging Technologies*

Received date: 31 May 2018
Revised date: 21 November 2018
Accepted date: 6 January 2019

Please cite this article as: Carlos A. Pinto, Ana P. Martins, Mauro D. Santos, Liliana G. Fidalgo, Ivonne Delgadillo, Jorge A. Saraiva , Growth inhibition and inactivation of Alicyclobacillus acidoterrestris endospores in apple juice by hyperbaric storage at ambient temperature. Innfoo (2018), https://doi.org/10.1016/j.ifset.2019.01.007

Research note

Growth inhibition and inactivation of *Alicyclobacillus acidoterrestris* endospores in apple juice by hyperbaric storage at ambient temperature

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Abstract

Control of endospores of *Alicyclobacillus acidoterrestris* in pasteurized apple juice using hyperbaric storage at 18 to 23°C was compared to storage at atmospheric pressure and 18 to 23 °C as well as refrigeration at ~4°C for up to 30 days. The juice samples were inoculated with approximately 1 x 10^5 CFU/mL spores. The juice spoiled quickly at atmospheric pressure and ambient temperature while under refrigeration spore levels were unchanged for 30 days. Hyperbaric storage of inoculated apple juice at 25, 50 and 100 MPa at 18 to 23°C resulted in spore inactivation at more rapid rates as pressure magnitudes increased reaching levels below the detection limit of 10 CFU/mL at 50 and 100 MPa. In highly acid foods such as apple juice, hyperbaric storage at pressures ≤100 MPa and ambient temperature was effective in inactivating spores of *A. acidoterrestris* for periods up to 30 days.

These results indicate hyperbaric storage at ambient temperature as a clearly more efficient preservation procedure to control the development of *A. acidoterrestris* endospores, compared to ambient temperature and refrigerated storage, in highly acidic foods as apple juice.

Keywords: *Alicyclobacillus acidoterrestris*; endospores, hyperbaric storage; highly acidic foods

1. Introduction

When it comes to the food industry, agriculture alone is responsible for up to one-third of the greenhouse effect gases releases to the atmosphere; refrigeration is considered the third major source of CO_2 **(Gilbert, 2012)**. In fact, about 1% of the worldwide emissions of CO_2 are attributed to refrigeration facilities in supermarkets **(James and James, 2010)**, thus, environmentally friendlier food preservation strategies are needed without jeopardizing food safety and quality.

Hyperbaric storage is a new concept of food storage under mild pressure that applies pressure as a means to preserve food products, which varies from the conventional refrigeration that employs temperature control, which is energetically expensive and environmental harmful. When done at ambient temperature, hyperbaric storage presents several advantages over refrigeration, such as reduced energetic costs (0.2¢ instead of 3.4¢ for refrigeration), as well as a lower carbon foot-print (0.0042 $kgCO_2/kg$ against 0.1085 $kgCO_2/kg$ for refrigeration) **(Ana Bermejo-Prada, Colmant, Otero, & Guignon, 2017)**, since energy is only needed during the compression and decompression phases of the pressure vessel (mainly the former). When the desired storage pressure is reached, all the valves are closed and the pressure is kept for long periods of time without energy costs **(Fernandes et al., 2014)**.

The feasibility of hyperbaric storage for the preservation of highly perishable food products was recently assessed, namely on ready-to-eat meals **(Moreira, Duarte, et al., 2015; Moreira, Fernandes, et al., 2015)**, raw bovine meat **(Freitas et al., 2016)**, watermelon juice **(Lemos, Ribeiro, Fidalgo, Delgadillo, & Saraiva, 2017; Pinto et al., 2017; Santos et al., 2015)**, among others, with the outcomes pointing toward shelf-life extensions by inhibition and/or inactivation of the endogenous vegetative microorganisms of the aforementioned products, as well as the general preservation of physicochemical attributes. For example, a recent study published by **Lemos, Ribeiro, Fidalgo, Delgadillo, & Saraiva, (2017)** showed the possibility of preserving raw watermelon juice (a highly perishable food) for at least 58 days while kept under pressure (62.5 MPa) at 15 ^{o}C.

When it comes to acidic food products, a study conducted in strawberry juice (pH 3.3) showed that hyperbaric storage (at ambient temperature) at 25 MPa slowed down microbial growth, while at 50 MPa and above microbial inactivation was observed **(Bermejo-Prada, López-Caballero, & Otero, 2016)**. A storage pressure of 50 MPa was

not suitable for preservation of watermelon juice due to microbial growth **(Lemos et al., 2017; Pinto et al., 2017)** (in fact, hyperbaric storage at 50 MPa (at ambient temperature) performed similarly to refrigeration to preserve watermelon juice, i.e. the juice spoiled similarly at HS conditions and refrigeration at atmospheric pressure). These differences between strawberry and watermelon juices stored at 50 MPa at ambient temperature might be related to the acidity of the strawberry juice that in combination with pressure contributed to the microbial load reductions observed at 50 MPa.

An even more recent study with bacterial spores from our group showed the possibility of preventing the germination of *Bacillus subtilis* endospores in three nutritional different matrices (McIlvaine buffer, carrot juice and brain-heart infusion (BHI) broth, all at pH 6.0), while stored at 50 and 100 MPa at 18 to 21 ºC for 60 days. In fact, the spore levels were reduced during storage, reaching the quantification limit of 2.00 log CFU/mL in carrot juice and BHI broth at 50 and 100 MPa, pointing towards the possibility of extending the shelf-life of pasteurized low acidic food products (such as carrot juice), by avoiding endospore germination and outgrowth when compared to the conventional refrigerated storage; a storage pressure of 25 MPa allowed endospore germination and outgrowth, resulting in juice spoilage **(Pinto, Santos, Fidalgo, Delgadillo, & Saraiva, 2018)**.

Compared to low-acidic foods, pasteurized acidic foods have an extended shelf-life due to the low pH that inhibits microbial growth. When it comes to very acidic food products (pH< 3.7), such as apple juice and carbonated beverages, conventional pasteurization eliminates the vegetative forms of pathogens, while endospores may remain but cannot undergo the germination process due to the pH. Consequently these products are shelf-stable at ambient temperature. Nevertheless, there are atypical cases of endospores that are able to germinate and outgrow under extreme acidic conditions, as is the case of *Alicyclobacillus acidoterrestris* **(Steyn, Cameron, & Witthuhn, 2011; Walker & Phillips, 2008)**.

A. acidoterrestris is a non-pathogenic, moderately thermophilic, acidophilic and spore-forming bacterium, whose occurrence in highly acidic fruit juices is common and expected **(Heyndrickx, 2011)**, representing a concern among fruit juice producers. Its presence is mainly due to unwashed or insufficiently washed fruit surfaces, since *A. acidoterrestris* is a soil-borne bacterium whose endospores are quite heat-resistant. In fact, *A. acidoterrestris* spores present D-values of 65.6 min at 85 ºC and 11.9 min at 91 ºC, in orange juice (pH ≈ 3.5), 57 min at 85 ºC and 16 min at 90 ºC for grape juice (pH ≈

3.3), or 56 min at 85 ºC and 2.8 min at 95 ºC for apple juice (pH ≈ 3.5) **(Silva, Gibbs, Vieira, & Silva, 1999)**, and 11.1 min at 90 ºC (z-value of 8.5 ºC) **(Bahçeci and Acar, 2007)**, which are higher time/temperature binomials than those commonly employed on juice pasteurization processes by the industry before the discovery of this spore-forming bacterium on highly acidic juices in Germany, in 1984 **(Cerny, Hennlich, & Poralla, 1984)**. These endospores are able to germinate and outgrow after a heat shock (typically 86 ºC to 96 ºC for 15 seconds to 2 min **(Lee, Dougherty, & Kang, 2002)**, or 80 ºC for 10 min, according to the **International Federation of Fruit Juice Producers, (2003)**), in a range of pH values of 2.5–6.0 and temperatures between 20 and 60 ºC, resulting in high cellular densities that spoil highly acidic fruit juices **(Heyndrickx, 2011)**, causing production of guaiacol (2-methoxyphenol) that is responsible for antiseptic off-flavours and odours **(Witthuhn, Smit, Cameron, & Venter, 2013; Walker & Phillips, 2008)**, as well as sediment deposition, cloudiness increase and discoloration in some juices **(Tianli, Jiangbo, & Yahong, 2014)**. These off-flavours, a result of guaiacol production, are a consequence of the metabolization of ferulic acid, and its derivatives, such as vanillin and vanillic acid, by *Alicyclobacillus* spp. such as several strains of *A. acidoterrestris*, *A. acidiphilus* and *A. herbarius* **(Goto et al., 2007; Smit et al., 2011)**. In the case of *A. acidoterrestris* (strain DSM 3922) bacteria are able to produce guaiacol at 25 ºC, which is quite a lower temperature than the optimum growth temperature (43-45 ºC) **(Corli Witthuhn et al., 2013)**. The most affected food products by *A. acidoterrestris* spoilage are pasteurized apple and orange juices and concentrates **(Steyn et al. 2011; Kumar et al. 2013)**, although **Silva et al. (1999)** reported its presence on high-acidic carbonated drinks and vegetable products, which, due to the low pH can be kept at ambient temperature.

High-pressure processing (HPP) itself alone does not inactivate endospores of *A. acidoterrestris* and even when combined with moderate temperatures (60 ºC), it is possible to achieve ≈3-log CFU/mL of reduction, as observed by **Vercammen et al. (2012)**, who reported no significant differences between unprocessed samples (phosphate buffer at pH 4 and 5, and citric acid buffer at pH 7) and processed samples (up to 600 MPa) for 10 min at ambient temperature, showing that the pH did not influence the inactivation rates in these buffers. The authors also reported no endospore reductions in tomato sauce (pH 4.2) at 600 MPa (10 min at ambient temperature); however, when the processing temperature was increased to 60 ºC, ≈1.5 log CFU/mL of endospore inactivation was observed, similarly to the inactivation level observed for the same

product at pH 5, which means that in this pH range, the most important factor concerning endospore inactivation is temperature, inasmuch the matrix pH, as well the nutritional composition (nutrient-free matrix as buffers *versus* tomato sauce). Similar results were found by Uchida and Silva, (2017), who reported ≈1.2 log CFU/mL *A. acidoterrestris* endospore inactivation at 600 MPa, 55 ºC for 5 min in malt extract broth (10 ºbrix, pH 3.8), with similar inactivation values when the treatment time was extended to 10 and 45 min, and the treatment temperature decreased to 45 and 35 ºC, respectively.

Bermejo-Prada et al., (2017) evaluated both energy and environmental associated costs (as aforementioned) regarding the possible implementation of the concept of hyperbaric storage at ambient temperature in an industrial unit. Generally, the integration of hyperbaric storage on the industry would be expensive due to the equipment costs (similarly to high-pressure processing), as it is a new concept of food preservation, there is no equipment available on the market specifically designed for hyperbaric storage, which would be potentially cheaper than the ones available for high pressure processing due to the lower pressure levels required to preserve food. Additionally, the vessel containing the juice could be moved within the warehouse or factory with a forklift, and the juice working as a pressurization fluid itself, as long as it regards a pumpable juice. The authors also found that the energy costs would be lower than conventional refrigeration, as the pressurized vessels could be transported to foreign geographies. The needless temperature control for hyperbaric storage allows considerable energetic savings, as well as a smaller carbon food-print, being an environmental-friendlier alternative to preserve food products.

Thus, in order to evaluate the potential of hyperbaric storage at ambient temperature to control the germination and outgrowth of *A. acidoterrestris* endospores in a high acidic food product (commercial pasteurized apple juice), hyperbaric storage at 25, 50 and 100 MPa was studied and compared with ambient and refrigerated storage (4ºC) (at atmospheric pressure) for 30 days. To do so, commercial apple juice was inoculated with *A. acidoterrestris* endospores and stored at the aforementioned conditions.

2. Materials and methods

2.1. Culture media and reagents

The *Bacillus acidophilus* agar (BAT agar) **(Vercammen et al., 2012)**, potato dextrose broth (PDB), potato dextrose agar (PDA) (all at pH 3.9 – 4.1), plate count agar

(PCA) (pH ≈7.0) and physiological solution (0.9% NaCl) were purchased from Applichem Panreac (Darmstadt, Germany) and sulphuric acid was bought from Sigma-Aldrich (Seelze, Germany).

2.2. Commercial apple juice

The commercial pasteurized apple juice (pH 3.50, 10 ºbrix) was purchased at a local supermarket and was used directly for inoculations, since preliminary microbiological analyses on general (PCA) and specific (BAT agar) culture media (for *A. acidoterrestris*) showed absence of microorganisms in the commercial juice.

2.3. *A. acidoterrestris* endospore preparation

A. acidoterrestris ATCC 49025 (DSM-3922) was purchased from *Deutsche Sammlung von Mikroorganismen und Zellkulturen* (DSMZ, *Braunschweig*, Germany). This microorganism was grown in BAT-agar, using the spread-plate method, at 45 ºC for 5 days, and afterwards a single colony was isolated and a liquid culture was grown in PDB that was incubated at 45 ºC for 2 days with shaking at 150 rpm. Then, the liquid culture was spread-plated (0.1 mL) onto PDA plates and kept at 45 ºC for 5 days **(Witthuhn et al., 2011)**, followed by a routine checking of the sporulation state of the bacteria, taking 20 days to achieve more than 95% of bright-phase endospores. Afterwards, the endospores were harvested by flooding the cultures with cold (4 ºC), sterile distilled water, and by scratching the agar plates with a bend glass rod, and then washed three times with cold, sterile distilled water by centrifugation (10 min at 5,000 ×*g* at 4 ºC), as performed by **Reineke et al. (2013)**. The washed endospores were stored in distilled water and kept in the dark at 4 ºC until use.

2.4. Endospore inoculations

Aliquots (2.7 mL) of commercial apple juice were aseptically placed in UV light-sterilized, low permeability polyamide–polyethylene, bags (PA/PE-90, Plásticos Macar – Indústria de plásticos Lda., Palmeiras, Portugal), using a laminar flow cabinet (BioSafety Cabinet Telstar Bio II Advance, Terrassa, Spain) to avoid contaminations. Then, 300 µL of *A. acidoterrestris* endospore suspension were inoculated on each matrix, at a final concentration of about 10^4-10^5 cells/mL.

2.5. Storage conditions

The storage experiments were carried out at 25, 50 and 100 MPa for 30 days at naturally variable ambient temperature (≈18-23 ºC), in a high pressure equipment (SPG13900, Stanstead Fluid Power, Stanstead, United Kingdom), with a vessel volume of 400 mL, using a mixture of propylene glycol and water (40:60 v/v) as the pressurization fluid. Simultaneously, two control samples were kept at atmospheric pressure and ambient temperature and at 4 ºC, submersed in the same pressurization fluid and kept in the dark. The adiabating heating was estimated from literature data to be from 2 to 4 ºC per 100 MPa **(Paredes-Sabja et al., 2007)**.

2.6. Endospore germination and inactivation

To assess both germinated and ungerminated endospores at each storage condition, an aliquot of the commercial apple juice (non-heated samples containing both vegetative cells and endospores, since germination and outgrowth might have occurred) was heated at 80 ºC for 20 min to inactivate the vegetative bacteria and heat-sensitive endospores (partially germinated), as performed by **Reineke et al. (2013)** and **Terano et al. (2005)**. With this procedure, total endospore load (TEL) was quantified while using non-heated samples allowed enumerating the total microbial loads (endospores and vegetative cells, TML). Then, decimal dilutions were performed (1.0 mL of each sample for 9.0 mL of physiological solution, 0.9% NaCl) that were spread-plated in BAT-agar and incubated at 45 ºC for 5 days **(Porebska, Sokołowska, Skapska, & Rzoska, 2017)**.

The results were expressed as the decimal logarithm variation (log (N/N_0)), obtained by the difference between the microbial load at each storage day (N) and the initial microbial load (N_0). The plates were considered as countable in a range of 1-150 colonies, thus, the detection limit (1.00 log CFU/mL) was considered when no colonies were found in the plates.

2.7. Statistical analyses

All the microbiological analyses were performed in triplicate, each one from duplicated samples. The results were statistically analysed using one-way Analysis of

variance (ANOVA), followed by Turkey's honest significand differences (HSD) test at 5% of significance, and were expressed as mean ± standard deviation.

3. Results and discussion

The initial loads of the commercial apple juice inoculated with *A. acidoterrestris* endospores is presented in **Table 1S**, showing some small differences between the different runs, since the experiments could not be carried all at the same time and several inoculations had to be done.

At ambient temperature (atmospheric pressure), both TML and TEL (unheated and heated samples, respectively) increased gradually about 1 log unit until the 5th day of storage **(Abel-Santos, 2014)**, as seen in **Figure 1**. Further microbiological analyses regarding this storage condition did not take place due to severe sample spoilage caused by microbial development.

Storage under refrigeration inhibited the development of the TML, that remained statistically similar ($p>0.05$) to the initial value along storage (except on the 30th day where a small decrease was observed), while the TEL load faced a small but statistically relevant ($p<0.05$) increase until the 5th day, and then decreased slightly ($p<0.05$) until the 30th day.

Contrarily to atmospheric pressure storage, hyperbaric storage caused both TML and TEL inactivation at all storage pressures. When kept at 25 MPa, the commercial apple juice presented gradual reductions ($p<0.05$), at least until the 5th day of storage, of both TML and TEL (2.40 and 2.20 log units, respectively), being statistically similar ($p>0.05$). Then, by the 20th day, the microbial load stabilized (> 2 to 3 log units reduction) and remained similar ($p>0.05$) for TML and TEL until the end of the storage experiments (**Figure 1**). Even though the present study regards an acidic commercial apple juice and a different endospore, **Pinto, Santos, Fidalgo, Delgadillo, & Saraiva, (2018)** reported that at 25 MPa, *B. subtilis* endospores (in carrot juice, pH 6.0) were not inactivated as verified in the present study (in fact, at 25 MPa, *B. subtilis* endospores were able to germinate and outgrow). This suggests that this pressure level is unable to hurdle spores' germination and growth and also that food products with lower pH's might need lower pressure levels than products with higher pH's (more perishable) to be preserved by hyperbaric storage. Nevertheless, more fundamental research in this field is needed to fully understand the effect of pH on endospore germination under pressure.

A higher inactivation rate was observed at 50 MPa, when compared to juice samples kept at 25 MPa. After 1 day at 50 MPa, a TML and TEL reduction of ≈ 2.00 and ≈ 2.20 log units was observed (**Figure 1**). Then, a linear decay of the TML and TEL was observed, (Log (N/N_0) = - 0.4093*(storage period (days)) - 1.6221), R^2 = 0.985) and (Log (N/N_0) = - 0.2367*(storage period (days)) - 1.9932), R^2 = 0.979), respectively. By the end of the storage experiments, both TML and TEL were below the detection limit (1.00 log CFU/mL), showing that hyperbaric storage at 50 MPa caused a higher inactivation effect on *A. acidoterrestris* in apple juice, compared to 25 MPa. Interestingly, for another acidic fruit juice under hyperbaric storage at 50 MPa, such as strawberry juice (pH 3.3), **Bermejo-Prada, López-Caballero, & Otero, (2016)** reported that a storage pressure of 25-50 MPa slightly inhibited endogenous vegetative forms proliferation, while for watermelon juice (pH 6.5) and raw bovine meat (pH 5.4), a storage pressure of 50 MPa was not effective to control microbial proliferation **(Freitas et al., 2016; Lemos et al., 2017; Pinto et al., 2017)**. When compared to carrot juice (pH 6.0) inoculated with *B. subtilis* endospores, hyperbaric storage at ambient temperature (at 50 MPa) avoided not only endospore germination and outgrowth but also caused endospore inactivation **(Pinto, Santos, Fidalgo, Delgadillo, & Saraiva, 2018)**, which might be related to the higher pH in this case.

The inactivation rate observed at 50 MPa was even more pronounced when the storage pressure was set to 100 MPa, since after 2 days of storage, the TML reached the detection limit, which had already been reached by the TEL after one day of storage, as seen in the **Figure 1**, with no further changes until the end of the storage experiments. Similar results were found by **Pinto, Santos, Fidalgo, Delgadillo, & Saraiva, (2018)** for *B. subtilis* endospores inoculated in carrot juice, even though the inactivation rates were slower than in the present study (after 30 days of hyperbaric storage at 100 MPa there were still quantifiable endospore counts in carrot juice), suggesting that the pH of the food product played a major role on microbial development/inactivation when foods are stored under hyperbaric storage conditions. In another study, **Sokołowska et al. (2015)** studied the influence of hydrostatic pressure treatments (100 MPa/20 minutes) combined with mild temperatures (50 ºC) on the germination and inactivation rates of *A. acidoterrestris* in commercial apple juice (pH 3.4 and 11.2 ºbrix). The results showed endospore germination and inactivation rates of ≈3.1 and ≈1.9 log CFU/mL, respectively. Both germination and inactivation rates increased to ≈3.6 and ≈2.0 log CFU/mL when the pressure level was 200 MPa, and decreased for pressures above 200 MPa. These results

reinforce the combined effect of low pressures (up to 200 MPa that triggers the nutrient-like germination pathway), the nutrients of commercial apple juice (that triggers the nutrient germination pathway) and temperature of 50 ºC (close to the optimum temperature of the cortex lytic enzymes (≈ 43 ºC) involved in the germination process) **Reineke et al. (2013)** to increase the germination and inactivation rates observed at the aforementioned study.

Even though conventional refrigeration was feasible to avoid endospore germination and outgrowth, hyperbaric storage at all studied pressures (25, 50 and 100 MPa) equally precluded (during 30 days) plus inactivated *A. acidoterrestris* endospores. Interestingly, for highly acidic food products as apple juice, the fact that *A. acidoterrestris* spores can outgrow at ambient temperature is a problem for these products. Nevertheless, under hyperbaric storage conditions cells cannot grow and are inactivated at pressure levels as low as 25 MPa, and even to undetected levels at 50 and 100 MPa. This is an interesting novel approach to control not only *A. acidoterrestris* endospores' development in acidic products, but also the development of other spores able to germinate and outgrow in acidic products. More research is necessary to further explore this potential.

4. Conclusions

All of the evaluated storage pressures were suitable to prevent spore outgrowth of *A. acidoterretris* with the extent of inactivation more pronounced as pressure increased. Hyperbaric storage at ambient temperature outperformed refrigeration since for the latter spore levels remained unchanged during storage. In conclusion, hyperbaric storage proved to be a feasible food storage methodology to control the development of *A. acidoterrestris* endospores, which are a problem to the juice industry, and, consequently, for consumers themselves.

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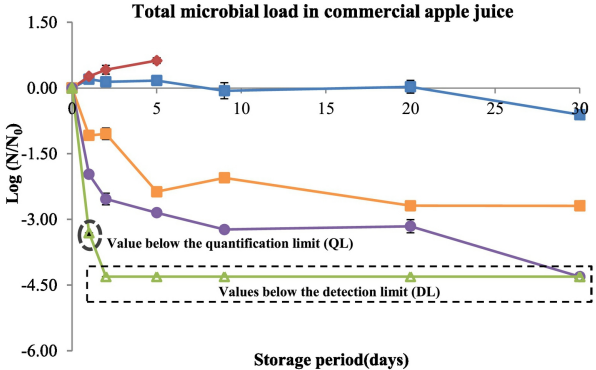
Highlights

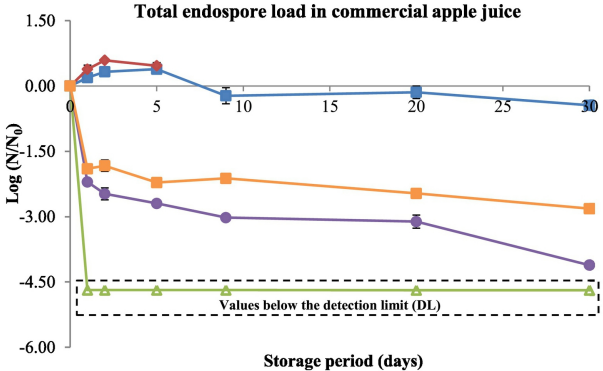
- Hyperbaric storage (HS) at 18 to 23°C inactivated ~5-log CFU/mL of *Alicyclobacillus acidoterrestris* spores at 50 and 100 MPa in apple juice over 30 and 2 days, respectively

- Spore inactivation rate increased with pressure magnitude (~2 log CFU/mL at 25 MPa for 30 days, ~5 log CFU/mL at 50 MPa for 30 days, and ~5 log CFU/mL at 100 MPa for 2 days at ambient temperatures

- Spores outgrew/remained unchanged at 0.1 MPa and ambient temperature /refrigeration, respectively

- HS/RT is a promising method to control *A. acidoterrestris* spores in highly acidic foods





AP/RT AP/RF 25 MPa/RT 50 MPa/RT 100 MPa/RT

Condition/ Storage period (days)	1	2	5	9	20	30
AP/RT	aDε	abD	bE	-	-	-
AP/RF	cDε	bcDε	bcDε	bDε	bcDε	aC
25 MPa/RT	dC	dC	bC	cC	aC	aB
50 MPa/RT	dB	cB	cB	bB	bB	aA
100 MPa/RT	bA	aA	aA	aA	aA	aA

Condition/ Storage period (days)	1	2	5	9	20	30
AP/RT	aD	bE	bE	-	-	-
AP/RF	cDε	cD	cD	abDε	bDε	aD
25 MPa/RT	cC	cC	bC	bcC	bC	aC
50 MPa/RT	dB	cB	cB	bB	bB	aB
100 MPa/RT	aA	aA	aA	aA	aA	aA

Figure 1