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Effect Of High-Pressure Thermal Sterilization On The Inactivation Of*Geobacillusstearothermophilus*Spores In Ready To Eat Meals

Ramos S.J. *, Millán, D., Ortiz, L., Alonso, D., García de la Torre, S.
Centro Nacional de Tecnología y Seguridad Alimentaria (CNTA), Carretera NA-134, km 53. 31570-San Adrian, Navarra, Spin
Corresponden Autor:Ramos S.J.

---ABSTRACT---
High-pressure thermal sterilization (HPTS) process is a promising technology for the production of high quality low-acid food products which are shelf-stable at room temperature. However, few studies have been conducted on the HPTS inactivation of bacterial spores in different low-acid food matrices. Therefore, within the EU FP SFS-17-2014 nº 635643founded Hipster project HPTS treatments at 600 MPa at 110, 115 and 121 ºC were performed on peas with ham, steamed sole, vegetable cream, and braised veal inoculated with G. stearothermophilusspores. HPTS treatments (600 MPa, 110 ºC, 5 min; 600 MPa, 115 ºC, 3 min and 600 MPa, 121 ºC during the come-up time) of food matrices allowed the achieving of more than 5-log$_{10}$ cycles of inactivation of most resistant G. stearothermophilus strains. The complex food matrices had a slightly protective effect on the inactivation of G. stearothermophilusspores during HPTS. The comparison of HPTS resistance with heat resistance at an equivalent process temperaturedemonstrated thesynergistic effect of both technologies. This means that high-pressurethermal processing can be carried out at lower temperatures and in a shorter time than conventional thermal processing to obtain similar inactivation levels. The results provide useful information onG. stearothermophilusspores for validating HPTS-processed low-acid foods.
KEYWORDS:*Geobacillusstearothermophilusspores High-pressure thermal sterilizationReady to eat mealsThermal treatmentsLow-acid foods*

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I. INTRODUCTION AND LITERATURE SURVEY

The traditional thermal process is the most widely applied method in the food industry forstabilizing foodstuffs microbiologically such as low-acid food products (Spilimbergo et al., 2002). However, the holding times and high temperatures required have a negative impact on the organoleptic and nutritional values of foods. For this reason, new emerging technologieshave been developedin recent years in order to achieve the microbial safety of the thermal process and to minimize the impact on the quality of the final food product.The concept of high-pressure processing (HPP) as a sterilization tool has been with us for several years.The inactivation of spores of the major bacterial spore-forming pathogens concerning us here is of particular interest (and concern) for low-acid shelf-stable foods. These pathogens are proteolytic strains of the neurotoxigenic species *Clostridium botulinum*.In the canning industry, commercial sterilization is achieved by a thermal process targeting a 12-log reduction in the*C. botulinum* spore population (an equivalent process lethality of about 3 min at 121 ºC) (Pflug, 1978). To achieve commercially viable sterility, the HPP sterilization process should result in a similar or improved inactivation of spores that is achieved by thermal processing. Prior to becoming a commercialprocess, the pressure resistance of bacterial spores of the pathogenicand spoilage type needs to be evaluated (Margosch et al., 2006).Moreover, the HPP process should be verifiable by biological validationto ensure the desired log cycle reductions of resistant non-pathogenicsurrogate spores (Koutchma et al.,2005; Sizer et al., 2002).Until now implementation for high-pressure thermal sterilization (HPTS) has not been developed (Heinz and Knorr, 2005; Hielmqwist, 2005; Juliano et al., 2009;Matser et al., 2004; Reineke et al., 2013a), whereas HPP as a pasteurization method has been available on the world market for the last 20 years (Cheftel, 1995; Hogan and Kelly, 2005; Patterson, 2005; Ramaswamy, 2011).Although no commercial units are currently operating, pilot scale systems are available and have been used to demonstrate that HPTS can work as analternativetechnology for thermal sterilization (Barbosa-Canovas and Juliano, 2008) and can become a feasibleand promising tool for the production of low-acid food products (Aoudhai et al., 2013; Koutchma et al., 2005; Lenz et al., 2014, 2015; Ramaswamy and Shao, 2010; Reddy et al., 2006; Sevenich et al., 2013,2014; Shao and Ramaswamy, 2011; Zhu et al., 2008).*Clostridium*spores(*Clostridium botulinum*, *Clostridium* *sporogenes*and *Clostridium perfringens*)and Bacillus spores (*Bacillus amyloliquefaciens* and*Geobacillusstearothermophilus*) are mentioned by numerous researchers as being highly resistant to pressure and temperature (Ahn and Balasubramaniam, 2007; Ahn et al., 2014; Juliano et al., 2009; Lenz et al., 2014, 2015; Paredes-Sabja et al., 2007; Ramirez et al., 2009; Reineke et al., 2013a; Wimalaratne and Farid, 2008). Furthermore, itis still unclear whether the potential surrogates for thermal processing can be used for validating HPTS in low-acid foods. Some thermal resistant microorganisms are pressure sensitive, whereas others sensitive to temperature are pressure resistant(Nakayama et al., 1996).

To overcome the limitations of non-thermal and thermal methods of food preservation, the HPTS process has been designed by combining pressure and temperature with the applying of lower intensities, but with equivalent or even higher degrees of stability and safety. HPTS technology combines the synergistic effect of high temperatures (90-121 ºC) and pressures to 400-600 MPa for an improved overall inactivation of spores and pathogenic microorganisms in addition to the retention of the food structure (Knoerzer et al., 2007; Matser et al., 2004; Sevenich et al., 2013; Sommerville, 2009). Therefore, the product needs to be pre-heated to 70-90 ºC, and by internal compression heating during a pressure build-up an instantaneous temperature increase is developed at up to 90-130 ºC. Depending on the food system this temperature increase can range from 3 to 9 ºC per 100 MPa and in addition helps to heat up the product to the required temperature. The main advantage of HPTS is that it accelerates spore inactivation in low-acid mediumsto shorten heating times due to compression heating and the synergistic effect of pressure and temperature (Barbosa-Canovas and Juliano, 2008; Knoerzer et al., 2010; Matser et al., 2004; Sevenich et al. 2013). In recent years much research has been conducted in order to comprehend the underlying mechanisms in the HPTS inactivation of spores, but many of them have been carried out at pressures and times not applied on an industrial scale (Ahn et al., 2014; Ramaswamy et al., 2010; Ratphitagsanti et al., 2010). Several authors have proposed a combined process of 600 MPa and 90-121 ºC to achieve economical holding times (≤10 min) by HPTS (Balasubramaniam, 2009; Koutchma et al., 2005; Margosch et al., 2004; Mathys et al., 2009; Rajan et al., 2004; Reineke et al., 2013a; Sevenich et al., 2013; Wimalaratne and Farid, 2008).Spore inactivation under high-pressuretemperature conditions is a two-step mechanism (Margoschet al., 2004a;Mathyset al., 2007; Reinekeet al., 2012, 2013a; Wuytacket al., 1998). At pressures above or equal to 600 MPa, the release of dipicolinic acid (DPA) from the spore core occurs and the spore starts to germinate and therefore becomes thermo and pressure sensitive and can be inactivated (Reinekeet al., 2013b; Setlow, 2003).Another important aspect is that the food system itself may have a protective effect on the spores because spores and microorganisms can interact with certain ingredients (fats, proteins, sugars, salts, etc) which then might lead to a retarded or incomplete inactivation (Olivier et al., 2011). This is why the application of HPTS needs to be tested on real food systems to ensure the safety of this process (Welti-Chanes et al., 2005). However, few studies have been conducted on the HPTS inactivation of bacterial spores suspended in various low-acid food matrices treated to a combination of pressure and temperatures.

Geobacillusstearothermophilus sporesareextremely heat resistant (up to 20 times more resistant than *C. botulinum)* (Ghani et al., 2001) and usually cause flat sour of canned foods. Because of the heat resistance of the spores of this microorganism, they are often used as a biological indicator to evaluate the effectiveness of thermal sterilization processes (Watanabe et al., 2003).Therefore, the aim of this study was to investigate: (i) the efficacy of HPTS on the inactivation of spores of *G. stearothermophilus*inoculated into several commercially available readyto eat (RTE) food systems: peas with ham (PH), vegetable cream (VC), steamed sole (SS), and braised veal (BV) and (ii) to define the P/T/tparameters to obtain 5-log reduction of *G. stearothermophilus* spores under industrially feasible treatment conditions. In thisstudy we compared the effects of heat, pressure, and HPTS on the inactivation of spores of *G. stearothermophilus* on ready to eat (RTE) meals.

II. RESEARCH ELABORATIONS

2.1 Preparation of bacterial and spore cultures

Four*G. stearothermophilus*strains CECT 43 (ATCC 12980), CECT 47, CECT 48, CECT 4517 (Spanish Type Culture Collection, Valencia, Spain)wereused. Furthermore, two wild strains isolated from braised veal and tomato soup treated at 500 MPa and 110ºC for 5 min were used in order to compare their resistance against non-wild spores. The bacterial cultures were kept frozen at -80^{o}C in cryovials. Strains were recovered from the cryovial by surface spreading on Tryptic Soy Agar supplemented with 0.6g/100 g. Yeast Extract (TSAYE) (Biolife, Italy) and incubated at 55^{o}C for 24 h. A broth subculture was prepared by inoculating a flask containing 10 mL of Tryptic Soy Broth supplemented with yeast extract (TSBYE) with one colony and incubated at 55^{o}C for 14-16 h. A volume of 50 mL of TSBYE was inoculated with 100 µL of subcultures and incubated at 55^{o}C for 14-16 h until the stationary stagewas reached (~1x10^{9}cfu/mL). Aliquots of 100 µL of the fresh culture were plated onto TSAYE agar plates and supplemented with $MnSO_4$(10 mg/L). Plates were incubatedat 55ºC for at least 10 days. The formation of endospores by *G. stearothermophilus*was confirmed by phase-contrast microscopy. The harvest was carried out when 95% of the spores were phase bright under the light microscope. The surface of the agar plates wasfloodedwith 20 mL of sterile distilled water and glass beads in order to harvest the spores. The solution obtained was centrifuged (5000g at 4ºC for 20 min) and five wash cycles in sterile distilled water were followed. A sonication process was performed for 10 min between the second and third wash to avoid clumping. The precipitate was re-suspended in sterile distilled water to give an initial viable spore count of ~10^8spores/mL, and the spore suspensions were pasteurized at 80 ºC for 10 min in order to inactivate remained vegetative cells. Spore suspensions containing >95% phase bright spores were stored at 4°C until treated (maximum 30 days).

2.2 Sample Preparation and inoculation

Green peas with ham, vegetable cream, steamed sole, and braised veal were obtained fromIndustriasAlimentarias de Navarra (IAN, Navarra, Spain) and the Marfo Food Group B.V. (Lelystad, Netherlands). The key ingredients of peas with ham were green peas, carrots, ham, olive oil, and salt, while those of vegetable cream were green peas, potatoes, onions, zucchini, olive oil, and salt. For braised veal the main ingredients were braised meat (veal, salt), potatoes, gnocchi, broccoli, mushrooms, cabbages, turnips, carrots, olive oil, salt, spices, herbs,and garlic and vinegar sauce, and forthe steamed sole RTE meal they were sole, carrots, onions, potatoes and vinegar, garlic, mustard, white wine, and cream of lime juice. The samples were selected as representatives of low-acid foods with different physico-chemical characteristics (Table 1). All RTE meals were prepared by food companies and supplied refrigerated prior to the sterilization process.

Table 1. Physico-chemical characteristics of different RTE meals

Nutritional Content (g/100g)	Vegetable cream	Peas ham	Braised veal	Steamed sole
Fat	2.90±0.41	12.8±0.36	6.23±0.41	8.62±0.27
Protein	1.20±0.15	5.11±0.26	7.63±0.32	10.2±0.56
pH	5.82±0.30	5.87±0.25	6.53±0.19	6.75±0.22
salt	1.00±0,10	1.10±0,21	3.69±0,29	2.95±0,36
a_w	0,963±0.004	0,982±0.002	0,972±0.001	0,987±0.003

Approximately $2x10^8$cfu/mL of *G. stearothermophilus* spores was inoculated in 10 g RTE meals. The inoculated samples were packaged in sterile PE/EVOH/PE bags (Papeles El Carmen, Navarra, Spain). The pouches were then heat-sealed under vacuum package conditions. One hour prior to use the samples were conditioned in a water bath at 25 ºC to regulate their internal temperature.

2.3 Measurement of physico-chemical parameters

Water activity (a_w) was measured at 25 ºC using a LabMaster system (Novasina AG, Pfaeffikon, Switzerland), which has a user-selectable internal temperature control. Each sample was consistently mixed in sterilized plastic bags in order to determine the pH using apHmeter (model GLP 21, Crison Instruments, S.A., Barcelona, Spain). The fat content was determined by the Soxhlet method (AOAC, 1990). The sodium content was analyzed by ICP/MS. Protein quantification was obtained by the Kjeldahlmethod.Values for a_w, pH, fat, salt and protein content are shown in Table 1.

2.4. High-pressure thermal sterilization treatment

The high-pressure equipment used was a discontinuous isostatic system from Stansted Fluid Power FPG 11500 B (Stansted, Essex, United Kingdom). With this unit pressuresup to 800 MPa and temperatures up to 130 ºC can be reached. The high-pressure transmitting medium was a mixture of propylene glycol (PPG)/water (70:30 v/v). The unit consisted of one chamber with a volume of 30 mL.The pressure build-up rate was 240 MPa/min and the pressure release time was less than 30 s regardless of the levels of target pressure.A circulating water bath was used to circulate temperature controlled PPG around the pressure vessel to regulate the shell temperature. In order to achieve the desired final process temperature, the initial temperatures of test samples were adjusted based on the compression heating factor (Fig.1).

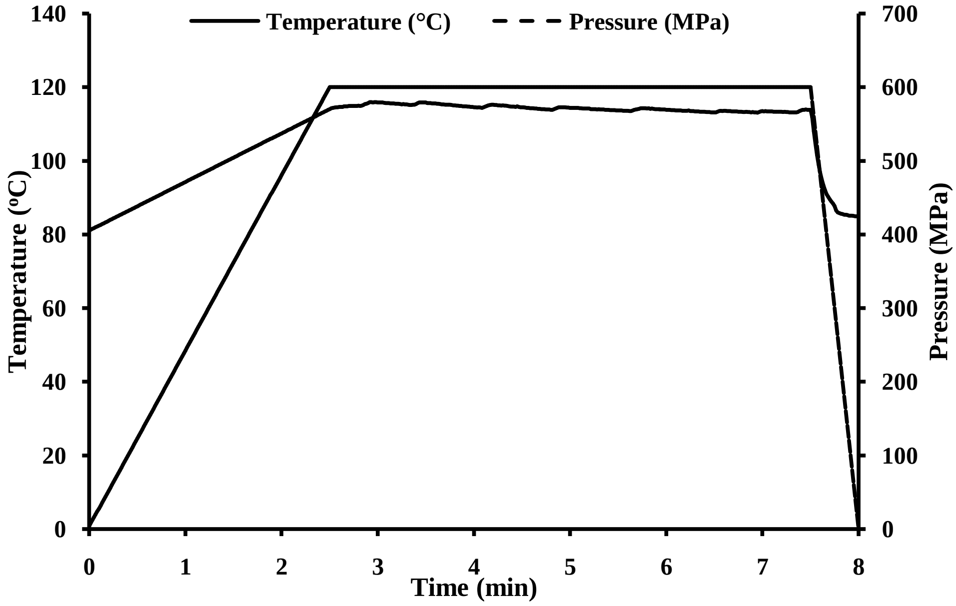

Figure 1. Typical pressure and temperature profile observed during pressure-temperature sterilization process

For this purpose, a control sample filled with each food product was put in a plastic cylinder vial equipped with a K-thermocouple (Stansted Fluid Power, Essex, UK) and placed in the geometrical center of the food sample. Subsequently pouches containing the inoculated food samples were preheated to the desired initial temperature using a water bath (1140 S, VWR International Eurolab S.L., Barcelona, Spain). The predetermined conditions using the above procedure are given in Table 2. The preheated samples were immediately loaded into a high-pressure chamber and subjected to pressure (500 or 600 MPa) and heat (110, 115or 121 ºC) for different hold time intervals (1, 3 or 5 min). The process hold time did not include the pressure come-up or depressurization times. The temperature data were recorded every second by using a data logger during the preheating, the pressure come-up, and the holding and depressurization times (Fig.1). Immediately after decompression, pouches were removed from the unit and samples were cooled immediately in an ice bath to avoid further inactivation.

Table 2. Experimental set up and temperature control for the HPTS tests.

System Pressure (MPa)	Target process temperature (ºC)	Required initial temperature (ºC)				Mean target Temperature Process (ºC)			
		VC	PH	BV	SS	VC	PH	BV	SS
500	110	80.91	82.53	83.12	83.03	109.54±0,27	109.88±0,36	109.69±0,98	109.76±0,53
600	110	72.16	73.13	74.14	72.02	109.68±0,47	109.80±0,63	109.75±1,05	110.01±0,46
600	115	79.87	80.08	81.14	80.25	115.22±0,63	115.04±0,80	114.77±0,38	115.27±0,38
600	121	85.24	86.14	87.02	86.98	121.09±0,39	120.84±0,56	121.45±0,30	120.66±0,42

HPTS conditions resulting in a 5-log reduction of endospores of *G. stearothermophilus*were selected for comparison with the equivalent thermal treatment. Furthermore, pressure treatments at 600 and 700 MPa at room temperature for 10 minwere conductedto determine the synergistic or additive effect of pressure and temperature, achieving < 0.5 log inactivation even at 700 MPa for 10 min in all the RTE meals tested (data not shown).

2.5. Thermal treatment

Heat treatments were carried out in a specially designed thermoresistometeras described by Condon et al. (1993). The thermoresistometerTR-SC is a mixing method designed for studying heat inactivation kinetics by the multipoint method.

Briefly, this instrument consists of a 400-mL vessel provided with an electric heater and thermostat controller for thermo-regulation, an agitation device to ensure inocula distribution and temperature homogeneity, and ports for sample injection and extraction. 350 mL of the samplewere placed in the vessel of the TR-SC and the heating was turned on. Once the treatment temperature hadachieved stability (110, 115 and 121 ± 0.2ºC), 0.2 mL of an appropriately diluted microbial cell suspension was injected into the vessel containing the food matrix. After inoculation, 0.2 mL samples were collected at different heating times and immediately pour plated.

2.6. Enumeration of survival spores

G. stearothermophilus spores treated by HPP and HPTS and suspended in vegetable creamwere directly diluted with 0.1% buffer peptone water and pour plated on TSAYE. On the contrary, peas with ham, braised veal, and steamed sole were blended for 2 min in a laboratory mix (Stomacher Macs 500 AES-Chemunex, Bruz, France) and serially diluted (1:10) in buffer peptone water. Dilutions of mixed slurries were pourplated on TSAYE. The plates were incubated at 55ºC for 48 h. Preheated spore samples were also enumerated to determine the effects on the initial spore population during the come-up time.

Temperature inactivation kinetics at a constanttemperaturewas analyzed using a first-order kinetic model:

$$\text{Log}\,(N/N_o) = -t/D \quad \text{(Eq. 1)}$$

in which N is the number of surviving spores (cfu/mL) after a heat treatment time of t (min); N_o is the initial concentration of spores (cfu/mL); and D is the decimal reduction time or D value which is the treatment time at any given temperature that will result in the destruction of 90% of the existing spore population (i.e. it results in one decimal reduction in the spore survivors). The D values were obtained from the linear regression slope of log (N/N_o) vs. t as negative reciprocal slopes (or on a semi-log plot the time taken to pass through a logarithmic cycle). To fit survival curves and to calculate resistance parameters the Geeraerd and Van Impe inactivation model-fitting tool (GInaFiT) was used (Geeraerd et al., 2005). Determination coefficient (R^2) values were also included to show the accuracy of the fitting. Since experiments were performed over several days, small variations in the initial concentrations were unavoidable. Inactivation was therefore expressed as the logarithm of the survivor fractions (N/N_o) under each condition and normalized to begin at a nominal initial concentration depending on the treatment. This helps to better compare the different survivor counts on the same plot. The detection limit was 10 cfu/g. The error bars in the figures indicate the standard error of the means for the data points obtained from at least three times on separate days.

III. RESULTS AND DISCUSSION

3.1. Pressure-temperature profiles

Figure 1 (showed before) shows the typical temperature changes in an example of a food matrix (braised veal) and a pressure chamber during HPTS treatments. For a treatment of 600 MPa and 115 ºC, the initial sample temperature was set at 81.14 ºC based on compression heating measurements (Table 2).After pressurization the temperature reached 114.02 ºCfollowed by a small increase to 1ºC due to the heat transfer from the chamber to the sample. The sample temperature was then stable (114.77 ºC±0.38) before the release of pressure after a 5-min holding time. Overall, it is easier to maintain the sample temperature constant during a short HPTS treatment. Based on this results it could be concluded that the sample temperature was effectively controlled and that quasi-isothermal conditions (± 1ºC) were achieved during holding time.

3.2. Variation in the resistance of different collection strains of G. stearothermophilusto HPTS

G. stearothermophilus is one of the main bacteria responsible for food spoilage and is both ubiquitous and spore forming. Variations in pressure and temperature resistance betweenfour *G. stearothermophilus*collectionstrains were studied in four RTE meals treated at 500 MPa and 110ºC at different holding times. The inactivation data for the different strains are given in Table 3.In the case of the four RTE meals, the CECT 48 strain was the most resistant independently of the time and food composition. Resistance variation between strains was checked at other HPTS combinations (600 MPa at 110 ºC and 600 MPa at 115 ºC) and the CECT 48 strain always proved to be the most pressure resistant strain. Based on these results, the CECT 48 strain was chosen for studying the effect of thermal and HPTS parameters on the resistance of this species.

Table 3. Viability loss (Log No/Nt) of four strains of *G. stearothermophilus* following combined treatment of pressure (500 MPa), temperature (110ºC) and time.

Strains	Log No/N reduction following HPTS at							
	Vegetable cream		**Peas ham**		**Braised veal**		**Steamed sole**	
	1 min	**3 min**	**1 min**	**3 min**	**1 min**	**3 min**	**1 min**	**3 min**
CECT 43	3.78	4.08	3.28	4.93	2.98	4.35	4.16	3.75
CECT 47	2.94	3.53	3.54	4.73	2.68	3.81	4.61	4.61
CECT 48	0.75	2.50	2.65	3.37	2.41	3.03	1.19	3.14
CECT 4517	2.55	3.40	3.06	3.88	3.57	3.84	3.49	4.67

3.3. Thermal destruction kinetics of G. stearothermophilus spores

Figure 2 shows the survivor curves for *G. stearothermophilus*CECT 48 spores under heat treatment conditions at 110, 115 and 121 ºC in the four RTE meals tested.

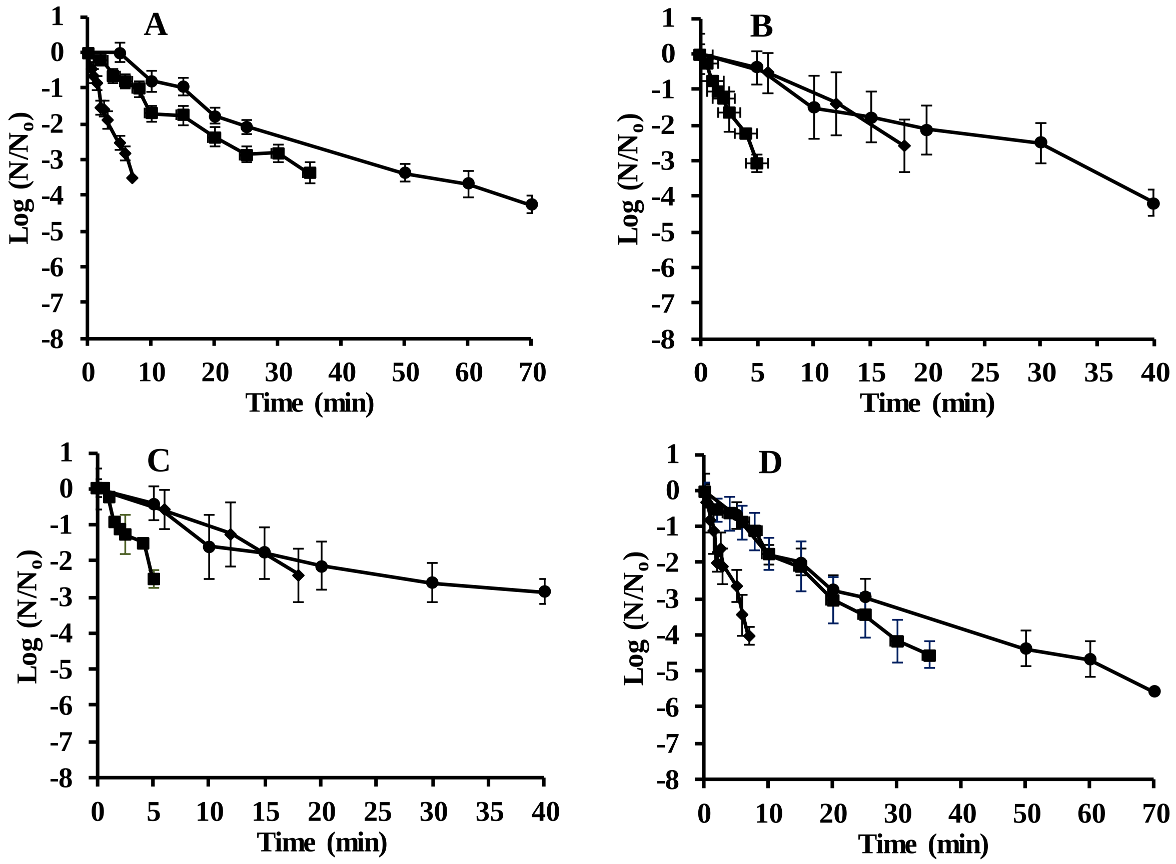

Figure 2. Survivor curves for *G. stearothermophilus* CECT 48 spores in thermally treated vegetable cream (A), green peas with ham (B), braised veal (C) and steamed sole (D) at selected temperatures: (●) 110 ºC, (■) 115 ºC and (♦) 121 ºC.

Table 4 summarizes the associated D and z values for thermal destruction kinetics. Higher temperatures resulted in higher rates of microbial inactivation and were represented by steeper survivor curves giving lower D values. Takinginto account a linear regression modelthe time necessary to inactivate 5-log (5D) can be calculated.Previous studies have shown that *G. stearothermophilus* spores are highly resistant to heat (Feeherry et al., 1987; Lopez et al., 1996, 1997; Periago et al., 1998; Watanabe et al., 2003).Feeherry et al. (1987) found D values when survivors were recovered on an antibiotic assay medium supplemented with 0.1% soluble starch to be 62.04, 18.00, 8.00, 3.33 and 1.05 min at 112.8, 115.6, 118.3, 121.1 and 123.9 ºC on *G. stearothermophilus* ATCC 12980, with a corresponding z value of 8.3ºC which indicates higher thermal resistance for ATCC 12980 spores. Watanabe et al. (2003) found that at 85 ºC the heat treatment D value for *G. stearothermophilus* ATCC 12980 spores was considerably higher than the values for four other species (*Bacillus coagulans*, *Bacillus subtilis, Bacillus cereus,* and*Bacillus licheniformis*). The ATCC 12980 strain corresponds to the CECT 43 strain of this study. This strainis commonly used in several heat and HPTS studies, but as described above its resistance to the combined process is lower than for other strains. This fact demonstrates the different resistance between strains and the need to include different strains in validation studies (Gayan et al., 2012).

Table 4. Decimal reduction time (D values) of *G. stearothermophilus* CECT 48 spores in RTE meals and thermal resistance Z_T values.

Temperature (ºC)	Vegetable cream			Peas ham			Braised veal			Steamed sole		
	D value (min)	R^2	Z_T (ºC)	D value (min)	R^2	Z_T (ºC)	D value (min)	R^2	Z_T (ºC)	D value (min)	R^2	Z_T (ºC)
110	16.7±0.27	0.93		15.2±2.89	0.97		15.9±0.31	0.95		14.1±0.53	0.94	
115	10.6±1.26	0.97	7.16	7.45±2.44	0.96	6.72	8.03±1.23	0.96	6.97	7.63±2,16	0.96	6.04
121	2.38±0.99	0.95		1.77±0.49	0.96		1.97±0.33	0.92		2.02±0.63	0.93	

2.7. HPTS destruction of G.stearothermophilus spores

As previously described, spores of *G. stearothermophilus* CECT 48 inoculated in different food systems were used to investigate the influence of HPTS. The trials were conductedat 600 MPa and 110, 115 and 121ºC (final temperature) under quasi-isothermal and isobaric conditions (Fig. 1). Figure 3 compares the spore survivors after HPTS treatments at 600 MPa at 110 and 115ºC for various treatment times.

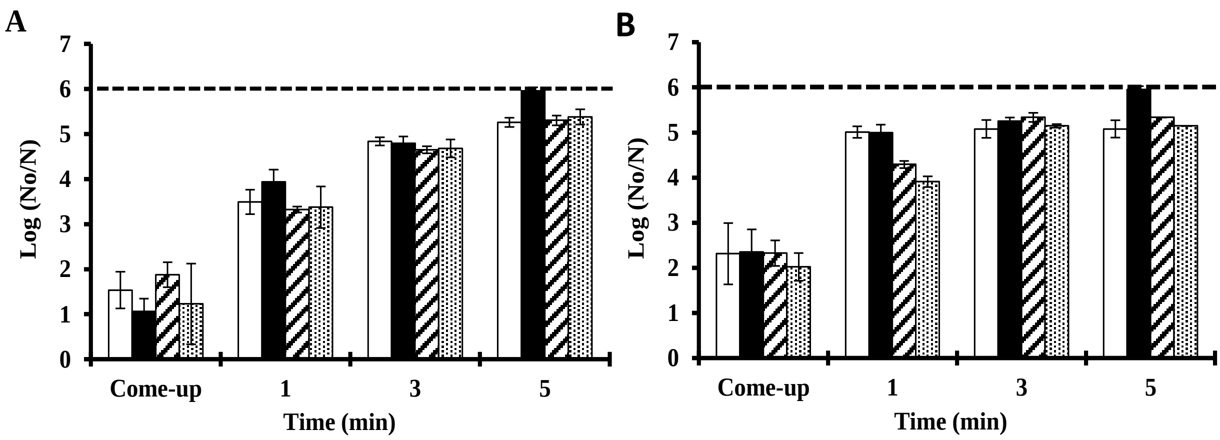

Figure 3. Effect of pressure and temperature on the inactivation of *G. stearothermophilus* spores in the tested food systems treated at 600 MPa, 110 ºC (A) and 600 MPa, 115 ºC (B). Vegetable cream (white square), steamed sole (black square), peas ham (stripped square) and braised veal (dotted square). The dotted horizontal line corresponds to the detection limit (10 cfu/mL).

During the HPTS come-up time (approximately 2.58 min), the reduction of *G. stearothermophilus* spores varied in different food matrices. The highest inactivation of *G. stearothermophilus* spores (1.88 Log cfu/mL) was observed in peas with ham, whereas steamed soleshowed the lowest reduction (1.01 Log cfu/mL) at 110ºC and 600 MPa during the come-up time (Fig.3.A). *G. stearothermophilus* spores in the four RTE meals were more susceptible to the combination of 115ºC and 600 MPa (approximately 2.3 Log cfu/mL) than those at 110ºC (Fig. 3.B). Furthermore, no differences at 115ºC were observed. At 121ºC and 600 MPa the inactivation of spores during the come-up time was similar to 5-log reduction in all the food matrices (data not shown), and the inactivation of spores was difficult to demonstrate because the inactivation takes place within the first few seconds of the treatment. This fact demonstrated that at 121 ºC it would not be necessary to apply holding times to achieve a 5-log reduction of *G. stearothermophilus* spores. These results suggest that the come-up timeisan important factor to be considered in spore inactivation.Several authors have reported different levels of spore reduction during the come-up time. Wang et al. (2009) described reduction levels of *B. coagulans* spores in milk and buffer during thecome-up time of 0.37 and 1.77 log at 400 MPa/80 ºC and 600 MPa/80 ºC, respectively.Ahnet al. (2007) reported significant reduction levels in different sporeforming bacteria, including *C. tyrobutylicum*ATCC 25755 (2.5 log), *T.thermosaccharolyticum*ATCC 27384 (2.1 log), *C.sporogenes*ATCC 7955 (3.3 log), *B. amyloliquefaciens*TMW 2479 Fad 82 (0.9 log), *B.amyloliquefaciens*TMW 2482 Fad 11/2 (0.8 log), *Bacillus sphaericus*NZ 14 (3.7 log), and*B.amyloliquefaciens* ATCC 49763 (2.5 log) at 700 MPa and 105 ºC. A reduction in*G. stearothermophilus* ATCC 12980 spores of 0.33 log and 1.23 at 500 MPa/80 ºC and 600 MPa/90 ºC, respectively in buffer during the come-up time was found whereas lower reductions were obtained in lu-wei beef (Wang et al., 2015).Ahn et al. (2014) also described the reduction level obtained during the come-up time in *G. stearothermophilus* spores in deionized water, cooked ground beef, egg patty mince, whole milk, and mashed potatoes at 105 ºC under 500 and 700 MPa.In this study the food matrices caused a protective effect on *G. stearothermophilus* spores during HPTS treatments. The highest inactivation (> 2 log) was observed in deionized water and the lowest reduction in cooked beef during the come-up time at 500 MPa and 105 ºC. Furthermore, spores in deionized water, whole milk, and mashed potatoes were more susceptible to the combination of 105 ºC and 700 MPa than those in cooked beef and egg patties.These observations suggest that different spores are likely to have different resistances during the pressure come-up time. Most of the studies of the combined effect of pressure and thermal treatment on microbial inactivation did not take into account the temperature increase in the samples during pressurization due to adiabatic heat (Wang et al., 2015).The temperature increase might significantly affect microbial inactivation results (Chen and Hoover, 2003). Therefore, compression heating during pressure treatment should not be ignoredand its contribution to processing lethality is sometimes considerable. Unfortunately,not all these data can be directly comparedbecauseexisting differences can vary depending on the high pressure-temperature system, target pressure, isothermal conditions, and the pressure pump (Ratphitagsantiet al., 2009; Wang et al., 2009).For this reason, reporting the pressure come-up time and the corresponding log-reduction during pressure-thermal treatment is important for accurate comparisons of different experimental results.

Comparing the inactivation effect of HPTS on the certified indicator of thermal sterilization, *G. stearothermophilus*, between 3-4 log inactivation at 110 ºC, 600 MPa were achieved for all systemstested within the first minute (Fig. 3.A.). Furthermore, the increase to a time of 5 minutes resulted in an inactivation of *G. stearothermophilus* spores of ≥ 5-log. When the process temperature was increased to 115 ºC, further enhancement in the spore lethality was observed (Fig. 3.B). The magnitude of spore reduction with increasing temperature was higher in vegetable cream and steamed sole after 1 min in comparison with peas with ham and braised veal. An increase of 1.51 and 1.06 log cfu/mL was observed in vegetable cream and steamed sole respectively while, an increase of 0.97 and 0.53 log cfu/mL was obtained in peas with ham and braised veal respectively at a process temperature increase from 110 ºC to 115 ºC.The increase to 115 ºC at 600 MPa, showed a shortening of the dwell time in vegetable cream and steamed sole to reach an inactivation of ≥ 5-log after 1 min. The inactivation of 5-log of *G. stearothermophilus*spores in peas with ham and braised veal at 115ºC at 600 MPa was possible after 3 min. An increasing temperature level can accelerate the inactivation of bacterial spores depending on food matrices and reduce the process time, which results in a cost-effective process. As stated by Reineke et al. (2012b), if the threshold pressure of 600 MPa is reached the driving force of the inactivation is the temperature.

The resistance of *G. stearothermophilus* CECT 48 spores to HPTS was significantly lower than that to thermal processing at an equivalent process temperature. It was observed that pressure treatment contributed to the acceleration of the destruction rate of *G. stearothermophilus*spores, which resulted in a significantly lower time to reach 5-log reduction (5D). At 115 ºC the associated 5D values were 53.0, 37.3, 40.2 and 38.2min in vegetable cream, peas with ham, braised veal, and steamed solerespectively, whereas 1-3 min are necessary to reduce 5-log by HPTS at 115 ºC and 600 MPa for four food matrices. On the other hand, a reduction level of≥ 5-log is achieved during the first seconds at 121ºC and 600 MPa while 5D inactivation was reached in 11.9, 8.85, 11.9 and 10.1 min at 121 ºC in vegetable cream, peas with ham, braised veal and steamed sole, respectively. This indicated that high pressure combined with temperature can inactivate bacterial spores more effectively and shortens processing times. Similar results have been reported when other spores (*Clostridium tyrobutyricym, C. thermosaccharolyticum, C. sporogenes, B. amyloliquefaciens and G. stearothermophilus*) were subjected to HPTS (Ahn et al., 2007; Margosch et al., 2004; Rajan et al., 2005, 2006; Ramaswamy et al., 2010;Rovere et al., 1996; Zhu et al., 2008). The synergistic effect (the lethality of the combined pressure was higher than the sum of the lethality of individual treatments) of pressure and temperature on the destruction of *G. stearothermophilus* spores was shown. For example, the 5D value at 110 ºC in braised veal was 79.5 min, whereas 5-log reduction was achieved in 5 min at 600 MPa and 110 ºC and no spore inactivation by HPP at room temperature was observed. This means that under high pressure thermal processing can be carried out at a lower temperature to obtain the same inactivation result, due to a synergistic effect of both technologies acting simultaneously

(Ramaswamy et al., 2010; Zhu et al., 2008). Furthermore, at a given temperature high pressure processing can be conducted in a much shorter time than conventional thermal processing. For example, in peas with ham the time to reach 5-log reduction of *G. stearothermophilus* spores at 115 ºC and 600 MPa was 12.4 times lower than with thermal treatment at the same temperature.

On the other hand,the food system itself can have a protective effect on the spores because certain ingredients such as fats, proteins, divalent cations (calcium and magnesium), sugar, salts, and the resultant water activity of the food can lead to retarded inactivation during pressure treatment (Ahn et al., 2014; Black et al., 2007; Garriga et al., 2004; Solomon et al., 2004). It has been shown that the composition of different treatment media (peas with ham, vegetable cream, steamed sole, and braised veal) did not have a protective effect on the inactivation of *G. stearothermophilus* sporesat 600 MPa and 110 ºC. However, a slightly baroprotective effect of the matrix was apparent at higher temperatures (T 115 °C). The results obtained in this study showed that *G. stearothermophilus*spores were effectively inactivated with a decreasing fat content in which the number of spores in 2.9, 8.6, 6.8 and 12.8% fat in vegetable cream, steamed sole, braised veal and peas with ham were reduced by approximately 5, 4.8, 3.91 and 4.29 log cfu/mL respectively at 115ºC and by 600 MPa for 1 min. Although peas with ham presented a higher fat content, spores reduction was lower in braised veal probably due to a fat-protein-salt combination related to a lower a_w in this product so as toprovide a protective medium for spore inactivation. Atlonger processing times (3 and 5 min) the relatively protective effect of the food systems seems to disappear.In contrast to our study, some authors have demonstrated a clearly food composition protective effect on the inactivation of spore forming bacteria (Ababouch et al., 1995;Ahn et al., 2014; Ananta et al., 2001; El Moueffak et al., 2001; Garriga et al., 2004; Heinz et al., 2001; Jung et al., 2012; Kruk et al., 2014; Sevenich et al., 2013, 2014, 2015; Solomon et al., 2004; Wang et al., 2015).Ahn et al (2014) observed that *G. stearothermophilus*spores inoculated in cooked beef were more resistant to the combination of pressure and temperature than in other food matrices such as egg patties, milk, and mashed potatoes. This observation may result from the relative high fat content (18.8 %) and low aw (0.93) of cooked beef in comparison with the other foods.These observations were similar to that suggested by Wang et al. (2015). The resistance of *G. stearothermophilus* spores was higher in lu-wei beef than in buffer solution. A reduction higher than 4 log units in the buffer at 500 MPa and 90 ºC for 15 min was obtained, whereas the reduction was lower (i.e. 3 log units) in lu-wei beef under the same conditions.The contents of fat and protein in lu-wei beef were higher which might have a protective effect on bacterial spores. The overall conclusion was that HPP or HPT preservation of fat/oil containing matrices could be morechallenging due to the formation of local (or global) low aw refuges. It is important to note also the importance of protein content in the food matrices to protect microbial inactivation by pressure. After pressure treatment at 300 MPa for 5 min, the level of inactivation of *Pseudomona aeruginosa* was 3.9, 3.4 and 2.3 $\log_{10}$ cycles in buffer, whey, and milk respectively (Ramos et al., 2015) due to the protection by the fats, proteins and divalent cations in whole milk that may protect cell membranes.

2.8. Effect of optimized HPTS treatments on RTE meals

Several authors have reported differences in response to HPTS between different species and between strains of the same species (Ahn et al., 2007; Lenz et al., 2014; Margosch et al., 2004, 2006; Olivier et al., 2011; Paredes-Sabja et al., 2007; Ramaswamy et al., 2013; Reddy et al., 1999, 2006; Sevenich et al., 2014) in different buffer systems and food matrices. Taking into account this consideration, the use of a single strain for the determinations of a specific treatment for a given log reduction is risky (Oteiza et al., 2010). Results previously shown demonstrated the different resistance of four collection strains of *G. stearothermophilus* spores. Furthermore, some authors have described a higher pressureresistance in wild strains isolated from some different foods than in the respective non-wild strains of the same species(Alpas et al., 1999; Benito et al., 1999).Therefore, the lethal effect of HPTS at 115 ºC and 600 MPa on *G.stearothermophilus* CECT 48 spores inoculated in peas with ham and braised veal (foods showing greater resistance) was performed to validate the designed combined treatment against two wild strains isolated from braised veal and tomato soup. Figure 4 comparesthe spore survivors of two wild strains after HPTS treatments in peas with ham and braised veal for various treatment times. Survivors of *G. stearotherrmophilus* CECT 48 in both food products have also been included for comparative purposes. As was observed, inactivation levels obtained in peas with ham and braised veal for the different strains were similar andno significant differences were found. Overall results demonstrated that in this case there were no differences between most resistant non-wild strains and wild strains, and that an HPTS treatment of 115ºC, 600 MPa for 3 min allowed more than 5 $\log_{10}$ cycles of inactivation of selected strains of *G. stearothermophilus* spores in low-acid foods with high fat and protein content.

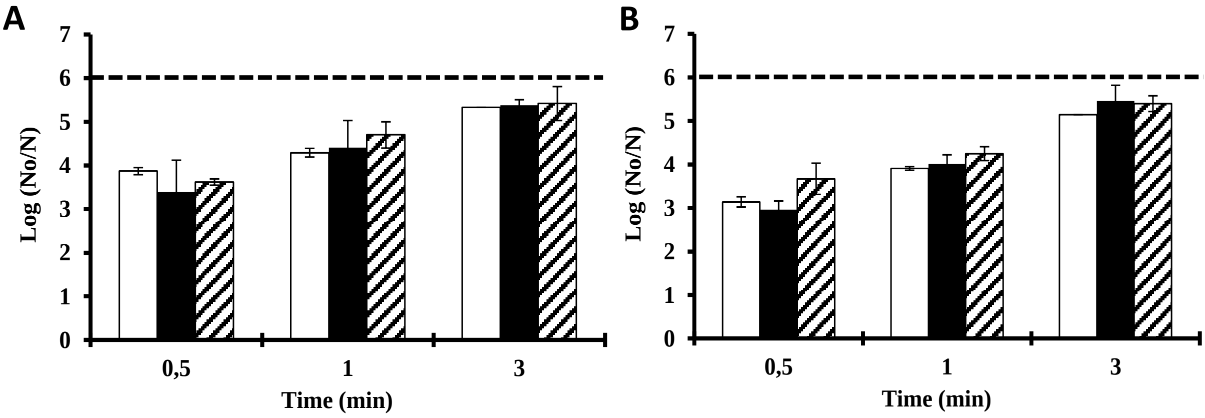

Fig. 4.*G. stearothermophilus*CECT 48 (white square), isolated from tomato (black square) and isolated from braised veal (stripped square) spores survivors inoculated in peas ham (A) and braised veal (B) and treated at 115 ºC and 600 MPa. The dotted horizontal line corresponds to the detection limit (10 cfu/mL).

IV. CONCLUSIONS

HPTS resistance can vary among strains of the same bacterial species. Four collection strains and two wild strains isolated from braised veal and tomato soup of *G. stearothermophilus*were evaluated. Most resistant strains were always provedto be non-wild CECT 48. Inactivation results of *G. stearothermophilus* CECT 48 spores were obtained under conditions allowing a temperature control for each HPTS treatment. Most of the studies on the combined effect of high-pressure thermal sterilization on bacterial spores did not consider the temperature increase in the samples duringthe come-up time due to adiabatic heating. Significant inactivation of *G. stearothermophilus*spores was obtained during the come-up time, even ≥ 5-log at 121 ºC, 600 MPa. The temperature increase might significantly affect the microbial inactivation results (Chen and Hoover, 2003a). Therefore, the inactivation during the come-uptime and its contribution to processing lethalityshould be taken into account. Results indicated that high-pressure thermal sterilization acts synergistically to allow the destruction of bacterial spores by using compression heating to instantaneoushigh temperatures, which results in much quicker processing than conventional thermal treatment, and economical dwell times (≤ 10 min) could have been reached with a pressure-temperature combination. 5-log reduction of *G. stearothermophilus*could be obtained at 110ºC, 5 min and 115ºC, 3 min at a maximum pressure of 600 MPa. The HPTS resistance of *G. stearotherophilus* spores varied slightly in different food matrices.In conclusion, the combination of high pressure and temperature is a potentially useful tool for inactivating*G. stearothermophilus*spores for low acid food sterilization. However, further study is needed to obtain accurate data on the HPTS inactivation of bacterial spores under different processing parameters and food compositionso as to provide practical information to predict spore inactivation by this technology and to evaluate its impact on quality characteristics.

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