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Inactivation of *Bacillus* spores inoculated in milk by Ultra High Pressure Homogenization

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ABSTRACT

Ultra High-Pressure Homogenization treatments at 300 MPa with inlet temperatures (*Ti*) of 55, 65, 75 and 85 °C were applied to commercial Ultra High Temperature treated whole milk inoculated with *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus sporothermodurans*, *Bacillus coagulans*, *Geobacillus stearothermophilus and Bacillus subtilis* spores in order to evaluate the inactivation level achieved. Ultra High-Pressure Homogenization conditions at 300 MPa with *Ti* = 75 and 85 °C were capable of a spore inactivation of ~5 log CFU/mL. Furthermore, under these processing conditions, commercial sterility (evaluated as the complete inactivation of the inoculated spores) was obtained in milk, with the exception of *G. stearothermophilus* and *B. subtilis* treated at 300 MPa with *Ti* = 75 °C. The results showed that *G. stearothermophilus* and *B. subtilis* have higher resistance to the Ultra High-Pressure Homogenization treatments applied than the other microorganisms inoculated and that a treatment performed at 300 MPa with *Ti* = 85 °C was necessary to completely inactivate these microorganisms at the spore level inoculated (~1 × 10^6 CFU/mL). Besides, a change in the resistance of *B. licheniformis*, *B. sporothermodurans*, *G. stearothermophilus* and *B. subtilis* spores was observed as the inactivation obtained increased remarkably in treatments performed with *Ti* between 65 and 75 °C. This study provides important evidence of the suitability of UHPH technology for the inactivation of spores in high numbers, leading to the possibility of obtaining commercially sterile milk.

1. Introduction

The thermal inactivation of *Bacillus* spores in commercially sterile milk through Ultra High Temperature (UHT) treatment has been extensively studied (Peleg et al., 2008; Scheldeman et al., 2006; Tabit and Buys, 2011). The latter is due to the high thermal resistance of the spores and their harmful effects on milk shelf life (Pacheco-Sanchez and Massaguer, 2007; Topcu et al., 2006). Among the *Bacillus* spores, the strains with the highest thermal resistance are *Bacillus sporothermodurans* (D_{140} = 3.4–7.9 s) and *Geobacillus stearothermophilus* (D_{140} = 0.9 s) (Huemer et al., 1998). These spores are able to survive the UHT treatment and then germinate in UHT milk, being able to grow in quantities that may exceed 1 × 10^5 CFU/mL (Aouadhi et al., 2012).

In an attempt to find similar treatments to the UHT process, a number of non-thermal technologies such as Ultraviolet (UV) irradiation, high hydrostatic pressure (HHP), pulsed electric fields (PEF), ultrasound, microwave heating, and ohmic heating have been used to eliminate spores in milk (Laguerre et al., 2011; Olivier et al., 2011; Shao et al., 2010; Somavat et al., 2012). These technologies have been investigated as they also reduce some of the disadvantages of the thermal process, such as loss of nutritional components, the development of cooked off-flavors and protein denaturation (Cattaneo et al., 2008; Datta and Deeth, 2003; Walstra et al., 2006).

Ultra High-Pressure Homogenization (UHPH) technology is a novel process for the treatment of food-liquid products. It has been applied in the chemical, pharmaceutical and biochemical industries, and recently in the food industry with interesting results. Based on traditional homogenization, the process features improvements to both the valve material, which helps to reach pressures up to 400 MPa, and valve geometry (Briñez et al., 2007; Diels and Michiels, 2006; Floury et al., 2004; Huppertz, 2011). The most important processing parameters are operating pressure, inlet temperature (*Ti*), and the number of passes (Diels and Michiels, 2006). The main mechanisms involved in the microbial

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inactivation by UHPH treatment are mostly mechanical such as shear stress, high speed collisions, impingement, cavitation and changes in internal energy (Datta et al., 2005; Diels and Michiels, 2006; Donsì et al., 2009; Paquin, 1999). Moreover, UHPH treatment has showed to be successful in obtaining pasteurized milk products, achieving a product with a 15-day shelflife (Pereda et al., 2007). Additionally, high microbial reductions in pathogen microorganisms such as *Listeria monocytogenes*, *Staphylococcus aureus*, and *Escherichia coli* have been achieved (Briñez et al., 2007; Donsì et al., 2009; Wuytack et al., 2002). Despite this, the application of UHPH to the inactivation of spores in milk and dairy products has been barely explored. Feijoo et al. (1997) inoculated spores of *Bacillus licheniformis* in ice cream and treated them at 50, 100, 150 and 200 MPa with *T*i of 33, 36, 40 and 50 °C; however, after treatments a less than 1 log CFU/mL spore reduction was achieved in certain conditions (200 MPa and *T*i = 50 °C). In the same way, Pinho et al. (2011) inoculated *Bacillus stearothermophilus* spores into skim milk and treated them at 100, 200 and 300 MPa with *T*i = 45 °C. Again, their results showed an inactivation of less than 1 log cycle in all cases, proving that *T*i has an important role in spore inactivation by UHPH.

Due to the pressure increase, an increment in the fluid temperature is expected, which occurs for a very short period of time if the appropriate heat exchangers are placed after the homogenization valve in the process, as necessary part of the UHPH process (Datta et al., 2005; Diels and Michiels, 2006; Dumay et al., 2013; Pereda et al., 2007). Moreover, the increment of *T*i has been demonstrated to improve the rate of microorganism inactivation in a synergic effect with the pressure applied, enhancing the effectiveness of the treatment (Diels et al., 2004, 2003; Dumay et al., 2013; Harrisson et al., 1991; Picart et al., 2006; Vachon et al., 2002). In this sense, Pathanibul et al. (2009) evaluated the effect of pressure and temperature by separate in UHPH treatments in the inactivation of *Listeria innocua* and *E. coli* inoculated in apple and carrot juices in the range of 50–350 MPa (no *T*i indicated), analyzing the thermal effect in the UHPH by applying heat treatments on the inoculated microorganisms. The results indicated that in UHPH treatments below 200 MPa, the inactivation obtained was mainly due to mechanical effects than the thermal mechanisms in the homogenization valve for both microorganisms and juices. However, when the fluid temperature in the HP-valve reached ~80 °C, the observed microbial inactivation could mainly be explained by the shear-induced temperature effects, even with the very short residence times. Therefore, with sufficient *T*i in UHPH treatments above 200 MPa, the UHPH process would reduce the quantity of bacterial spores sufficiently to obtain commercial sterile products. For these reasons, the aim of this study was to evaluate the inactivation of *Bacillus* genus spores in milk by applying UHPH technology with moderate inlet temperatures.

2. Materials and methods

2.1. Bacterial growing conditions and sporulation

Table 1 presents the different growing conditions applied to obtain the bacterial spores used in this experiment. To obtain the spore suspensions, an enrichment process was first carried out, incubating the culture for 48 h. Inocula of 2 mL of the culture were then spread in Roux flask containing the sporulation medium. The sporulation phase was carried out over 20 days, and monitored by phase contrast microscope (Euromex, Model F, Arnhe, Holland) until the sporulation medium presented 90–95% of spores.

2.2. Preparation of spore suspensions

Spores were collected by flooding the agar surface with sterile water (30 mL), with the suspensions then being placed in centrifuge tubes (45 mL). After collection, the suspensions were washed four times by centrifugation at 10 000 *g* for 20 min at 4 °C (Sigma 4k15, Sigma, Germany). The final suspensions were heat-treated at 80 °C for 30 min, in order to inactivate any vegetative-cells, and then stored at 4 °C until use. Prior to use, the spore suspensions were enumerated (~6-7 log CFU/mL) and divided in 10 mL tubes. Before UHPH treatments, a heat treatment in the spore suspensions (10 mL) was carried out at 80 °C for 30 min to inactivate the vegetative cells being then inoculated in 0.5 L of spore-free UHT commercial whole milk (3.6% fat, 3% protein and 8% SNF) before the UHPH treatments. UHT milk, instead of raw milk, was used in this study because the reduced amount of spores in this kind of milk, and in order to observe the effect of the UHPH process in the spores inoculated without the presence of other spores and/or microorganisms and the interactions between them.

2.3. UHPH treatments

The UHPH treatments were performed in a high-pressure homogenizer (Stansted Benchtop Homogenizator nG12500, Stansted Fluid Power Ltd., Essex, UK). The equipment comprised a high-pressure ceramic valve able to support 400 MPa, and a second pneumatic valve, located after the first in the process, able to support up to 50 MPa. The high-pressure system consisted of 2 intensifiers driven by a hydraulic pump. The flow rate in the homogenizer was approximately 8 L/h. In order to minimize the effect of temperature retention after treatment, spiral-type heat-exchangers were located behind the second valve (Guamis et al., 2010). The inlet temperature (*T*i), the temperature after the milk had passed through the homogenization valve (T_v), and the final milk temperature (T_f) were monitored throughout the experiment. UHT commercial milk was UHPH-treated under the following

Table 1
Growing conditions for *Bacillus* spore-forming bacteria employed for this study.

Microorganism	Incubation T (°C)	Enrichment media	Sporulation media	Enumeration media	Reference
B. cereus (CECT 5144)	30	TGB	MYA	GYA	AENOR, 2002
B. licheniformis (DSMZ 13)	30	TGB	MYA	GYA	AENOR, 2002
B. sporothermodurans (DSMZ 10599)	37	BHI	Campden	BHA + $MnSO_4 \cdot H_2O$	Periago et al. (2004)
B. coagulans (DSMZ 2356)	37	Nutritive broth	Nutritive Agar + $MnSO_4$	TSA + $MnSO_4 \cdot H_20$	Wang et al. (2009)
G. stearothermophilus (CECT 47)	45	TGB	MYA	GYA	AENOR, 2002
B. subtilis (CECT 4002)	37	TGB	MYA	GYA	AENOR, 2002

TGB = Triptone-glucose Broth MYA = Manganese-yeast agar GYA = Glucose-yeast agar BHI = Brain Heart Infusion broth BHA = Brain Heart Infusion Agar TSA = Tripticase-soy Agar.